THE UNIVERSITY *of* EDINBURGH

The Diversity of *Acinetobacter baumannii* isolates from Egypt

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Thesis presented
for the degree of Doctor of Philosophy
University of Edinburgh
2013

i. Abstract

Acinetobacter baumannii is an important nosocomial pathogen, frequently associated with morbidity and mortality in immunocompromised patients due to the immuno-ablative treatments, neutropenia and prolonged hospitalization. The ability of *A. baumannii* to survive in the healthcare setting makes it a frequent problematic pathogen in cancer centres. Much of the interest in *A. baumannii* has been attributed to its remarkable rapid acquisition of resistance mechanisms*A. baumannii* is an excellent example of genetic plasticity, with its ability to acquire and express resistance in plasmids and chromosome particularly to carbapenems

The aim of this thesis is to look at the molecular epidemiology and resistance mechanisms of 34 non-duplicate *A. baumannii* in two cancer centres in Cairo, Egypt.

Initial sequencing of the ubiquitous $bla_{\text{OXA-51-like}}$ gene revealed a large diversity within the strains, with eight different genes identified: $bla_{\text{OXA-64}}$, $bla_{\text{OXA-65}}$, $bla_{\text{OXA-66}}$, $bla_{\text{OXA-69}}$, $bla_{\text{OXA-71}}$, $bla_{\text{OXA-78}}$, $bla_{\text{OXA-94}}$, $bla_{\text{OXA-89/100}}$.

Typing with Pulsed-field Gel Electrophoresis (PFGE) showed an overall similarity at only 28.69% between the isolates, with variation in pattern for isolates with similar $bla_{\text{OXA-51-like}}$ genes.

Typing with Multilocus Sequence Typing (MLST) identified 6 new Sequence Types: ST408 - ST414, in addition to ST331 and ST108 which have been previously found in other regions of the world.

All three OXA-type carbapenemases: bla_{OXA23}, bla_{OXA40} and bla_{OXA58}, responsible for conferring carbapenem resistance were found in the collection studied. Insertion sequences IS*Aba1,* IS*Aba2*

and IS*Aba3* have been found to upregulate the expression of bla_{OXA} genes. IS*Aba1* was found upstream of bla_{OXA23} in 18 strains in this collection The first report of IS*Aba2* was identified upstream of a $bla_{\text{OXA-51-like}}$ gene in this collection. Additionally, IS*Aba3* was bracketing the bla_{OXA58} genes, and two isolates harboured hybrid promoters with IS*1006* and IS*1008* interrupting the upstream IS*Aba3* sequence.

Resistance to Ceftazidime was mediated by Extended-spectrum β-lactamase (ESBL) genes belonging to PER-like group: $bla_{\text{PER-1}}$, $bla_{\text{PER-7}}$ and the first report of $bla_{\text{PER-3}}$ gene and its genetic environment in *A. baumannii*.

In conclusion, this study shows the diversity exhibited by *A. baumannii* in Egypt. The various resistance mechanisms illustrate the ability of *A. baumannii* in acquiring and expressing resistance genes, either on plasmids or in the chromosome. Furthermore, the results indicate an urgent need to strict infection control policies and surveillance of antimicrobial use in Egyptian hospitals.

ii. Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Leena Al-Hassan

iii. Dedication

I dedicate this thesis to my parents, for their love and continuous support. You are my source of strength and I hope I made you proud.

iv. Acknowledgments

First and foremost, I want to thank God for the strength and courage to complete this study, as well as the many blessings I have in my life.

Second of all, I would like to thank my supervisor Professor Sebastian Amyes for the continuous support, the invaluable advice, and the flexibility he has given me in completing this work.

All my appreciation to the research department and the laboratory services at the Children's Cancer Hospital in Cairo for facilitating the work undertaken at their centre; particularly Prof. Dr. Hadir El Mehallawy, for her tremendous help throughout this thesis and in providing the samples.

My thanks to all my colleagues, and a special thanks and love for my dear friends for always being there for me.

Last but not least, all my love and appreciation for my family and husband for their continuous encouragement and love.

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vi. Publications and presentations

L. Al-Hassan, H. El Mahallawy & S.G.B. Amyes. (2012). First report of *bla*$_{\text{PER-3}}$ in *Acinetobacter baumannii*. *Int'l J of Antimicrob Agents*, **41**: 93-94.

L. Al-Hassan, H. El Mahallawy & S.G.B. Amyes (2012). Diversity in *Acinetobacter baumannii* Isolates from Paediatric Cancer Patients in Egypt. *Clin Microb Infect* (E-pub ahead of print on 15 Feb 2013).

B. Lopes, L. Al-Hassan & S.G.B. Amyes. (2012). IS*Aba825* controls the expression of the chromosomal *bla*$_{\text{OXA-51-like}}$ and the plasmid borne *bla*$_{\text{OXA-58}}$ gene in clinical isolates of *Acinetobacter baumannii* isolated from the USA. *Clin Microbiol Infect*, **11**:446-51.

L. Al-Hassan, H. El Mahallawy & S.G.B. Amyes (2012). Diversity in *Acinetobacter baumannii* Isolates from Paediatric Cancer Patients in Egypt. (Poster, ECCMID 2012, London, UK).

L. Al-Hassan, H. El Mahallawy, B. Lopes & S.G.B. Amyes. (2012). The identification of IS*1006*-like sequence interrupting an IS*Aba3* upstream of *bla*$_{\text{OXA-58}}$ in *Acinetobacter baumannii* from a cancer patient in Egypt. (Poster, ECCMID 2012, London, UK).

L. Al-Hassan, A. Opazo, H. El Mahallawy & S.G.B. Amyes. (2012). The Role of IS*6* Family Promoters of *bla*$_{\text{OXA-58}}$ in Conferring Carbapenem Resistance in *Acinetobacter baumannii*. (Under Review).

vii. Abbreviations

G+C	Percentage of DNA consisting of guanine and cytosine bases
ICU	Intensive Care Unit
UTI	Urinary Tract Infections
GIT	Gastrointestinal tract
VAP	Ventilator-associated pneumonia
NNIS	National Nosocomial Infections Surveillance
NCCN	National Comprehensive Cancer Network
LPS	Lipopolysaccharide
PBP	Penicillin-binding protein
OMP	Outer-membrane protein
ESBL	Extended-spectrum β-lactamase
MBL	Metallo β-lactamase
ADC	Acintobacter-derived cephalosporinase
CHDL	Carbapenem-hydrolysing class D β-lactamase
MIC	Minimum inhibitory concentration
PFGE	Pulsed-field Gel Electrophoresis
MLST	Multi-locus Sequence Typing
WW	World-wide Clone
IS	Insertion Sequence
PCR	Polymerase Chain Reaction
CR	Common Regions
NAMRU-3	Naval Medical Research Unit 3
CCH	Children's Cancer Hospital
NCI	National Cancer Institute
CLSI	Clinical and Laboratory Standards Institute
ST	Sequence Type

viii. List of tables and figures

Contents

Chapter 1: Introduction

1.1 The history of the genus *Acinetobacter*

The development of *Acinetobacter* spp. taxonomy was confusing and full of uncertainties. *Acinetobacter spp.* was initially described by Beijerinck in 1911 and assigned the name *Micrococcus calcoaceticus* (reviewed by Henriksen, 1973). The organism shared characteristics with *Moraxella* spp and was classified in the same group for some time; however significant differences were identified and led to the designation of this organism as *Achromobacter* spp (reviewed by Henriksen, 1973; reviewed by Peleg *et al*., 2008). *Acinetobacter* was then proposed by Brisou and Prévot in 1954 to distinguish the non-motile species in *Achromobacter* group (reviewed by Henriksen 1973). *A. calcoaceticus* and *A. lwoffii* were the proposed names given to the first described species and were distinguished by their ability, or not, to acidify glucose (reviewed by Peleg *et al.* 2008).

1.2 Taxonomy of *Acinetobacter*

Acinetobacter is defined as Gram-negative, strictly aerobic, glucose non-fermenting, non-motile, catalase-positive, oxidase-negative coccobacilli, usually occurring in diploid formation and with DNA G-C content of 39% to 47% (Giamarellou *et al.*, 2008; reviewed by Peleg *et al*., 2008).

DNA-DNA hybridization studies performed by Bouvet in 1986 identified 12 genospecies including *A. baumannii, A. calcoaceticus, A. haemolyticus, A. johnsonii, A. junnii* and *A. lwoffii* (reviewed by Peleg *et al.* 2008). Further work by Bouvet and Jeanjean, Tjernberg and

Ursing, and Neshmara *et al*. added more genospecies and resulted in the current genus *Acinetobacter* comprising 23 named species (Table 1) with the *A.calcoaceticus-A.baumannii* complex being closely related and the most clinically relevant (reviewed by Nemec *et al.* 2011; Peleg *et al*. 2008).

The *A. calcoaceticus-A. baumannii* complex is difficult to distinguish by conventional phenotypic methods and comprises *A. calcoaceticus*, an environmental organism, frequently isolated from soil and water (Henriksen, 1973; Nemec *et al*. 2011), *Acinetobacter* genomic species 3 (now referred to as *Acinetobacter pittii*), *Acinetobacter* genomic species 13 TU (now referred to as *Acinetobacter nosocomialis*) and *A.baumannii* which are implicated in nosocomial infections (Dijkshoorn *et al.*, 2007; Nemec *et al.*, 2011; Peleg *et al.,* 2008).

Species	Genomic Species	Source
A. calcoaceticus	1	Soil and humans
A. baumannii	2	Humans (clinical specimen), soil, meat and vegetables
A. pittii	3	Humans (clinical specimen), soil and vegetables
A. haemolyticus	4	Humans (clinical specimen)
A. junii	5	Humans (clinical specimen)
A. johnsonii	7	Humans (clinical specimen) and animals
A. lwoffii	8/9	Humans (clinical specimen) and animals
A. radioresistens	12	Humans (clinical specimen), soil and cotton.
A. bereziniae	10	Humans (clinical specimens), soil and vegetables
A.guillouiae	11	Humans (clinical specimens) and animals
A. nosocomialis	13TU	Humans (clinical specimen)
Acinetobacter ursingii		Humans (clinical specimens)
Acinetobacter schindleri		Humans (clinical specimens)
Acinetobacter parvus		Humans (clinical specimens) and animals
Acinetobacter baylyi		Activated sludge and soil
Acinetobacter bouvetii		Activated sludge*
Acinetobacter towneri		Activated sludge*
Acinetobacter tandoii		Activated sludge*
Acinetobacter grimontii		Activated sludge*
Acinetobacter tjernbergiae		Activated sludge*
Acinetobacter gerneri		Activated sludge*
Acinetobacter venetianus		Sea water
	13BJ, 14TU	Humans (clinical specimens)
	14BJ	Humans (clinical specimens)
	15BJ	Humans (clinical specimens)
	16	Humans (clinical specimens) and vegetables
	17	Humans (clinical specimens) and soil
	15TU	Humans (clinical specimens) and animals
	‘Between 1 and 3’	Humans (clinical specimens)
	‘Close to 13TU’	Humans (clinical specimens)
	13BJ, 14TU	Humans (clinical specimens)

Table 1: Classification of the genus *Acinetobacter* Adapted from (Dijkshoorn *et al*. 2007; Peleg *et al*. 2008; Nemec *et al*. 2011). *Activated sludge: process for treating sewage and industrial wastewaters.

1.3 Identification of *Acinetobacter spp.*

Acinetobacters have been previously assigned to the *Mimae* group due to the difficulty in de-staining hence misidentifying the organism either as Gram-positive or Gram-negative cocci (reviewed by Henriksen 1973; Peleg *et al*., 2008). *Acinetobacter* from human clinical samples grow well on conventional laboratory media at 37°C incubation, whereas species not belonging to the *A.calcoaceticus-A.baumannii* complex may not grow on McConkey agar (Nemec *et al*. 2011; Peleg *et al*. 2008).

Phenotypic detection methods and metabolic tests are not suitable for comprehensive identification of *Acinetobacter* and distinguishing it from other non-fermenting Gram-negative bacteria. They also do not allow the discrimination of the different genomic species. DNA-DNA hybridization scheme proposed by Bouvet and Grimont in 1986 assesses growth at 37°, 41° and 44°C, the ability to produce acid from glucose, gelatine hydrolysis, and assimilation of carbon sources, and this scheme identified 95.6% of 136 *Acinetobacter* isolates from human samples (reviewed by Peleg *et al*., 2008). However, this poorly discriminates between isolates belonging to the *A.calcoaceticus-A.baumannii* complex. Additionally, DNA-DNA hybridization and conventional phenotypic detection methods are not suitable for routine microbiology diagnostic laboratories due to the laborious nature of these methods. Laboratories hence rely mostly on automated and semi-automated machines such as Pheonix, Vitek, and MicroScan for identification and susceptibility testing, as they provide results much faster. Nevertheless, limitations still exist for using these machines in identifying acinetobacters, particularly the *A.calcoaceticus-A.baumannii* complex.

Molecular methods are subsequently much better tailored for accurate species identification of acinetobacters. These include amplified 16s rRNA gene restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP), ribotyping, tRNA spacer fingerprinting,

restriction analysis of the 16s-23srRNA intergenic spacer sequences, and sequencing of the *rpoB* gene (Dijkshoorn *et al.*, 2007; Dolzani *et al.*, 1995; Nemec *et al*., 2011; Peleg *et al*., 2008). More recently, the amplification and sequencing of the intrinsic $bla_{\text{OXA-51-like}}$ gene has also been widely used for identification of *A. baumannii* isolates (Evans *et al.*, 2008; Hamouda *et al.*, 2010; Woodford *et al*., 2006).

1.4 Natural habitat of *Acinetobacter* species

The natural habitat of *Acinetobacter spp* has been investigated extensively in the past few years and it is now considered a ubiquitous organism in nature. They can be recovered from soil, water, vegetables, animals and human body lice (reviewed by Peleg *et al.*, 2008; Giamarellou *et al*,. 2008; La Scola & Raoult, 2004). *A. johnsonnii, A. junnii* and *A. radioresistens* are also frequent colonizers of human skin and mucous membranes (reviewed by Peleg *et al.* 2008; Giamarellou *et al.* 2008). (Table 1)

Acinetobacter baumannii however is rarely identified as a commensal organism on human skin, and is not an environmental organism; so far we have failed to elucidate its natural habitat (Seifert *et al.* 1997). Despite being identified in the soil, the reports are not sufficient to provide conclusive evidence that *A. baumannii*'s natural habitat is soil.

Environmental wound contamination has also been investigated, as soldiers wounded in the Afghanistan, Iraq and Vietnam wars had a high rate of *A. baumannii* wound and blood infections (Hujer *et al*., 2006). Additionally, *A. baumannii* was isolated from human body louse from homeless people in France (La Scola & Raoult, 2004). *Acinetobacter* spp. including *A. baumannii* have also been isolated from vegetables (Berlau *et al.*, 1999). Overall, the results indicate the wide distribution of *A. baumannii* and may provide insight

into its potential natural habitat as well as providing a route for the dissemination into the hospital setting.

The hospital environment serves as a suitable habitat for *A. baumannii*, *A. pittii* (formerly known as geno.sp.3) and *A. nosocomialis* (formerly known as geno.sp. 13TU) (Nemec *et al.* 2011; Peleg *et al.* 2008; Dijkshoorn *et al*. 2007). These species are able to persist on inanimate surfaces for prolonged durations up to several months. Furthermore, the hospital environment provides a larger number of hosts that *A. baumannii* can infect. The moist conditions in hospitals, the room temperature together with the numerous devices it can contaminate, all provide ideal environments for *A. baumannii* to survive and disseminate.

1.5 Clinical manifestations of *A. baumannii* infections

A. baumannii infections are associated with increased morbidity and mortality as well as prolonged hospital stay (Metan *et al.*, 2009). However the mechanisms of virulence of *A. baumannii* are unclear (Gordon & Wareham, 2010). Biofilm formation is an important feature of *A. baumannii* as it readily adheres to inanimate surfaces and hence facilitate colonization and infection in the hospital setting (Gordon & Wareham, 2010; Peleg *et al*. 2008). Biofilms are difficult to get rid of as they are highly dense colonies of *A. baumannii* and exhibit high levels of resistance to disinfectants. Their persistence on inanimate surfaces increases the probability of transmission to patients by healthcare workers in addition to their spread.

1.5.1 *A. baumannii* in nosocomial infections

Patients at risk of developing *A. baumannii* infections are those with immunosuppression, prolonged hospitalization and critically ill patients in Intensive Care Units (ICUs) (Dijkshoorn *et al.* 2007). Nosocomial infections caused by *A. baumannii* include: pneumonia, bloodstream infections, skin and soft tissue infections, wound infections, urinary-tract infections (UTI) and, rarely, gastrointestinal tract (GIT) infections (Zarrilli *et al.*, 2009).

1.5.1.1 Hospital acquired pneumonia

Pneumonia, mainly ventilator-acquired pneumonia (VAP), is the most commonly identified clinical manifestation of *A. baumannii* (Dijkshoorn *et al*. 2007; Peleg *et al*. 2008). This could be attributed to the colonization of airways by *A. baumannii* which soon develop into true pneumonia upon prolonged hospital admission and extensive administration of antibiotics. Furthermore, data from the National Nosocomial Infections Surveillance (NNIS) System in USA show that ICU VAP rates due to *A. baumannii* reached 7% in 2003 (Falagas & Kopterides, 2006). A recent study by Lee *et al*. (2012) was aimed to study the different clinical manifestations associated with *A. baumannii* vs *A. nosocomialis* in pneumonia and revealed that *A. baumannii* pneumonias seem to be more severe and more likely to have associated abnormal haematological findings. The higher resistance rates observed in *A. baumannii* resulted in administration of inappropriate therapy as well as a higher mortality rate (Lee *et al.*, 2012).

1.5.1.2 Blood stream infections and sepsis

Bloodstream infections, particularly sepsis, is common in *A. baumannii* infections worldwide (Dijkshoorn *et al.*, 2007; El-Mahallawy *et al.*, 2005; Munford, 2006). In the USA, *A. baumannii* was found as the 10th most common aetiologic agent in nosocomial blood-stream infections (reviewed by Peleg *et al.* 2008). *A. baumannii* bacteraemia can be secondary to pneumonia, and can also result from central-venous line catheters, which act as a main route for dissemination of organisms into the blood stream (Simon *et al*. 2000). In a study performed on paediatric cancer patients in Cairo in 1999, *Acinetobacter* accounted for 6.7% of the total bloodstream infections in one centre (El-Mahallawy *et al*. 2005).

1.5.1.3 Skin and soft tissue infections

Skin, soft-tissue and wound infections caused by *A. baumannii* are increasingly reported and highlight the importance of environmental contamination in causing infections. Most reports are from wounded military personnel, as well as in burn patients (Adams-Haduch *et al.*, 2008; Hujer *et al*., 2006). Twenty-three soldiers wounded in the Iraq war of 2003 had *A. baumannii-A. calcoaceticus* wound infections, presented by different manifestations such as osteomyelitis, deep wound infections and burn infections (Davis *et al.*, 2005; Hujer *et al*., 2006). Such infections are not associated with high mortality rates but may be a source of bacteraemia and sepsis if inappropriate therapy is given.

1.5.1.4 Other manifestations

A. baumannii infections have also been reported in causing urinary tract infections (UTI) commonly associated with urinary catheters, as well as meningitis particularly with the

presence of an external ventricular drain (reviewed by Peleg *et al*. 2008). These infections represent less common clinical manifestations and occur due to the presence of external catheter-devices which in turn can be infected by contaminated hospital environments as well as by hospital personnel. Furthermore, air-borne transmission of bacteria plays a role in colonizing external catheters and hence providea a way of infections, particularly if the catheter is not sealed properly. Contaminated hands of the patients themselves or by their carers during the routine cleaning of the area around the catheter is also an important route of transmission.

1.5.2 Risk factors for developing *A. baumannii* infections

There are certain risk factors that predispose patients for infection with *A. baumannii*. These risk factors can occur simultaneously or separately, but contribute significantly to the morbidity and mortality observed with *A. baumannii* infections. Those include: surgery, trauma, underlying malignancy, previous administration of broad-spectrum antibiotics, previous admission to the ICU, exposure to contaminated equipment, mechanical ventilation, invasive procedures and presence of indwelling devices such as catheters (Falagas & Kopterides, 2006; Dijkshoorn et al. 2007; Giamarellou *et al*. 2008). Co-morbidities are associated with more severe *A. baumannii* infections that lead to unfavourable outcomes. It is however debatable whether mortality is due to the *A. baumannii* infection or is caused by the underlying co-morbidity. In a study by Turkoglu *et al.* (2011), *A. baumannii* was investigated in critically ill patients with haematological malignancies and despite the high rate of mortality in *A. baumannii* infections, it was not an independent risk factor for mortality. *A. baumannii* infections contributed, however, to longer ICU admission and prolonged duration of mechanical ventilation (Turkoglu *et al*. 2011).

1.5.3 Cancer-related infections

Infections in cancer patients are a common complication due to the immunoablative therapy as well as underlying illness (Santolaya *et al*., 2001). Febrile neutropaenia is a common medical emergency in cancer patients where fever is defined as a single oral temperature of 38.5° or greater, or two consecutive readings of 38.0° an hour apart, with neutropaenia defined as absolute neutrophil count (ANC) <500/mm^3 or <1000/mm^3 with an expected decline (Meckler & Lindemulder, 2009). Approximately 60% of febrile and neutropaenia episodes are caused by bacterial infections, and require the immediate administration of broad-spectrum antibiotics (Klastersky *et al.*, 2011). The epidemiology of pathogens, however, differs from one geographical region to another and also from one centre to another. Data from the USA show a predominance of Gram-positive pathogens, whereas in Taiwan Gram-negative pathogens predominate (Chen *et al.*, 2010). Data from Egypt also show a higher incidence of Gram-positive infections in paediatric cancer patients, but resistance to the first-line antibiotics such as amikacin and ceftazidime was much higher in Gram-negative pathogens; in addition to 50% of Gram-negative pathogens being resistant to the second-line antibiotic Imipenem (El-Mahallawy *et al.* 2005).

Bloodstream infections and central venous line infections are most commonly identified in paediatric cancer patients, particularly with haematological malignancies (Simon *et al*., 2008). In a study performed by Turkoglu *et al.* (2011) which investigated the characteristics of *A. baumannii* infections in patients with haematological malignancies, pneumonia was found as the most common infection site, with a higher mortality rate in patients with *A. baumannii*. The risk factors identified were similar to other clinical *A. baumannii* infections including ICU admission, prolonged hospitalization, presence of indwelling catheter devices, prior antibiotic therapy, underlying malignancy, intensified chemotherapy, neutropaenia, and immunosuppressive state (Santolaya *et al.*, 2001; Turkoglu *et al*., 2011).

The rise in antibiotic resistance is alarming in cancer patients, and has been attributed to the frequent antibiotic administration and the use of broad-spectrum antibiotics not only as treatment, but also as prophylaxis therapy (Saghir *et al.*, 2009). Monitoring of the susceptibility and cycling of the first-line antibiotics is therefore recommended in many cancer centres, and most importantly complying with the guidelines of antimicrobial use. The National Comprehensive Cancer Network (NCCN) have developed guidelines for the prevention and treatment of cancer-related infections, but individual centres should routinely monitor their antibiogram and prescribe antibiotics accordingly (NCCN Prevention and Treatment of Cancer-Related Infections, 2012).

1.5.4 *A. baumannii* in community-acquired infections

A. baumannii is rarely identified in community-acquired infections mainly due to its prevalence in the hospital setting. Reports have included community acquired pneumonia in patients with previous alcohol abuse with a mortality rate up to 60%. The source of the infection was attributed to throat carriage by individuals with excessive alcohol consumption (Anstey *et al.*, 2002; Falagas *et al.*, 2007). Community acquired *Acinetobacter* infections have been reported in tropical and sub-tropical regions and is commonly associated with a co-morbidity, such as chronic obstructive pulmonary disease, renal disease, diabetes and heavy smoking (Falagas *et al*., 2007).

1.5.5 Pathogenicity and virulence mechanisms of *A. baumannii*

Limited data is available on the pathogenicity and virulence mechanisms of *A. baumannii*; however, colonization with *A. baumannii* is more frequent than infection, which indicates a

relatively low pathogenicity when compared with other Gram-negative organisms (Dijkshoorn *et al.* 2007). Sixteen genomic islands carrying putative virulence genes associated with cell-envelope and biogenesis, lipid metabolism, iron uptake and metabolism, quorum sensing and a type IV secretion system as have been identified (reviewed by Dijkshoorn *et al*. 2007; Gordon &-Wareham, 2010). The ability of *A. baumannii* to adhere to biotic and abiotic surfaces allow formation of biofilms, which are mediated by pili. Pili thereafter interact with human epithelial cells and production of lipopolysaccharide (LPS) promotes adherence to the host cells (reviewed by Dijkshoorn *et al*. 2007; Gordon & Wareham, 2010). LPS also acts as an immunostimulatory factor leading to pro-inflammatory responses in *A. baumannii* infections (reviewed by Dijkshoorn *et al*. 2007).

Figure 1 gives a clear and detailed representation of the factors contributing to *A. baumannii* infections. Resistance to the bactericidal activity of the human serum is crucial for survival in the human host; in addition to quorum sensing and its role in biofilm formation. This consequently shows the ability of *A. baumannii* to survive and adapt to diverse environments, including the nutrient-scarce hospital setting and the human body (Dijkshoorn *et al*. 2007).

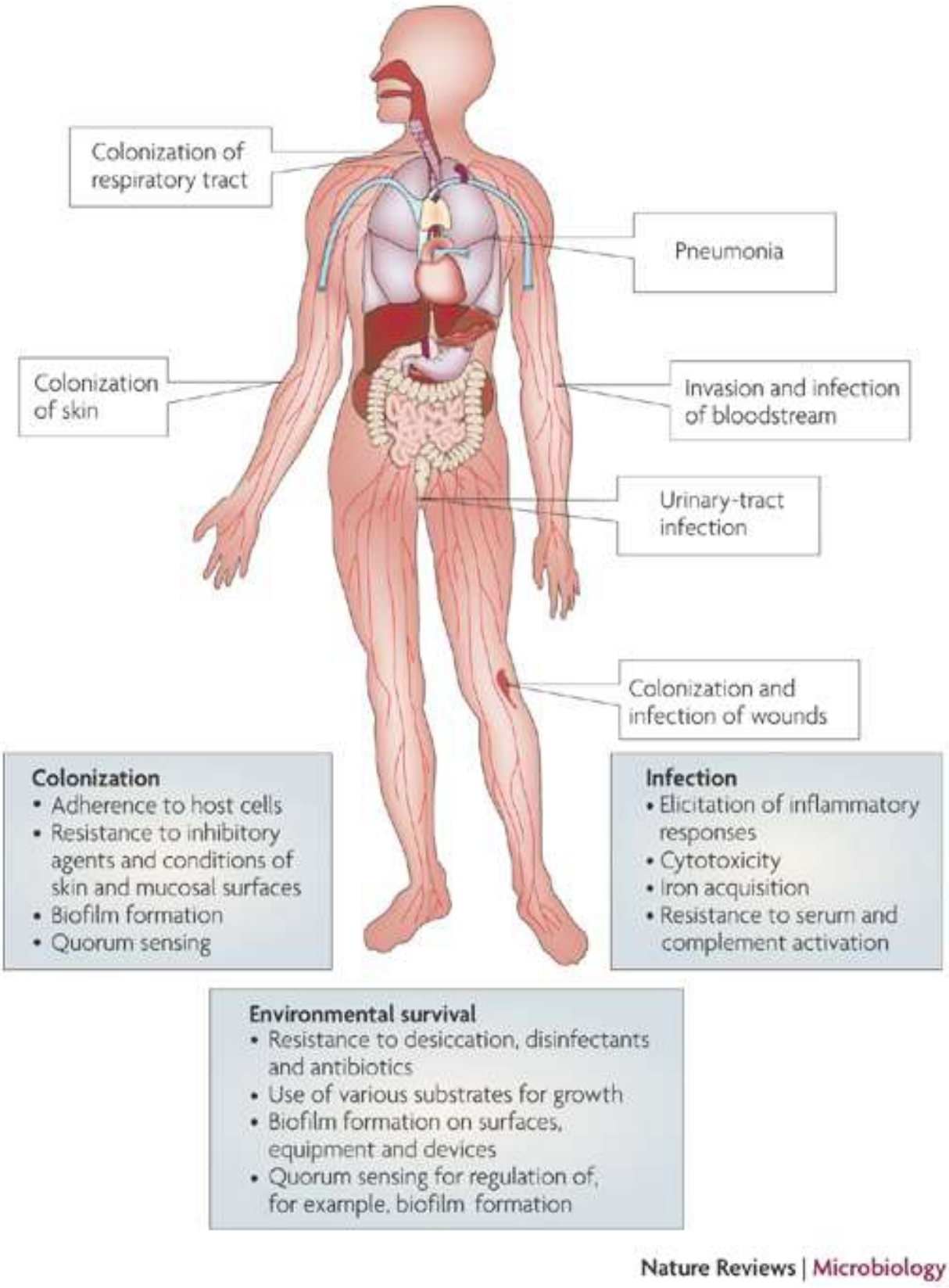


Figure 1: Factors contributing to *Acinetobacter baumannii* colonization and infection.
(Taken from Dijkshoorn *et al.* 2007)

Iron uptake is an important mechanism in *A. baumannii* infections. The ability of *A. baumannii* to obtain and utilise iron has contributed to its ability to survive both in the host and in the environment, which is mediated by the secretion of a variety of molecules involved in iron acquisition, including the siderophore acinetobactin, and also the production of a haemin utilisation system (Gordon & Wareham, 2010; Vallenet *et al.,* 2008).

Reduction in free extracellular iron concentrations by iron-binding proteins is one of the host defence mechanisms in response to bacterial infections (Vallenet *et al*., 2008). With the use of siderophores, *A. baumannii* is able to overcome this iron starvation by binding to host iron-binding proteins (reviewed by Dijkshoorn *et al.* 2007; Gordon & Wareham, 2010; Vallenet *et al.,* 2008).

Vallenet *et al*. (2008) studied the genomes of three *Acinetobacter* isolates: *A. baumannii* AYE, a multi-drug resistant strain involved in outbreaks in France (Fournier *et al.*, 2006), *A. baumannii* SDF, a susceptible strain isolated from human body louse (La Scola & Raoult, 2004) and *A. baylyi*, an environmental soil organism. Differences in the presence of pathogenicity genes revealed the different environmental conditions to which each bacterium is exposed. Strain AYE has the highest catabolic capacities, which are useful in surviving the nosocomial environments. Interestingly, siderophores were not identified in SDF indicating its ability to use haemoglobin as a source of iron. This could be due to the fact that SDF is in frequent contact with the gut of haematophage organisms and hence blood cells (Vallenet *et al*., 2008).

1.5.6 Recommended treatment of *A. baumannii* infections

The resistance patterns observed for *A. baumannii* in the clinical setting is leaving very few treatment options. Combination therapy is relied on in many centres to treat MDR strains of *A. baumannii*, where a significant synergy is observed *in vitro*. Combination therapy relies on an aminoglycoside with a 3rd generation cephalosporin, or colistin combined with rifampicin, ceftazidime or imipenem (Dijkshoorn *et al*., 2007; Gordon & Wareham, 2010). Combination therapy aims to prevent the emergence of resistance when using two different compounds, as well as provide coverage of a broad spectrum of pathogens in the case of mixed or unidentified infections (Legrand *et al.*, 2011; reviewed by Peleg *et al.*, 2008). Most importantly, antibiotic selection should rely on the susceptibility data of individual institutions (Peleg *et al.* 2008). Due to *A. baumannii* being inherently resistant to many antibiotics, carbapenems are the ideal drugs in treating *A. baumannii* infections, but resistance is emerging very rapidly, leaving few available treatment options (Knapp & English, 2001). Fluoroquinolones were used to treat sporadic cases of *A. baumannii*, but resistance is now widespread in endemic strains (reviwed by Dijkshoorn *et al*., 2007; Legrand *et al.*, 2011). The use of polymyxins and tigecycline has emerged in recent years to overcome carbapenem and multi-drug resistance, and have proven success in treating severe *A. baumannii* infections (Dijkshoorn *et al.*, 2007; Gordon & Wareham, 2010). Resistance is still relatively rare for these compounds; however the use must be closely monitored in order to monitor the emergence of resistance. The use of tigecycline for bloodstream infections is debatable as it is most effective in treating skin and soft-tissue infections, and its enhanced tissue penetration leads to serum concentrations below the pharmacodynamic breakpoint, resulting in recurrent bacteraemia and even aid in the emergence of resistance (Gordon & Wareham, 2009). Colistin (polymyxin E) has proven clinical efficacy in treating carbapenem and multi-drug resistant strains of *A. baumannii*, but the main issues of concern are

nephrotoxicity and heteroresistance (Perez *et al*. 2007; Peleg *et al.* 2008). Interestingly, colistin is combined with rifampicin or a carbapenem in treating metallo-β-lactamase producing *A. baumannii* (Legrand *et al*. 2011). Colistin is not available in most Egyptian hospitals and must be ordered from other countries to treat severe cases. Unfortunately, most Egyptian governmental hospitals lack the appropriate surveillance and monitoring of antibacterial consumption, so we are unable to get a clear indication of the most commonly used compounds.

Cancer patients are usually under prolonged antibiotic therapies due to neutropaenia. Antimicrobial therapy, and particularly carbapenems, may be administered for up to 10 days in a proven *A. baumannii* infection, and this increases the risk of development of resistance (Owens, 2008). This is why the resistance pattern is high in cancer centres, reaching up to 50% in some cases (Saghir *et al*., 2009; El-Mahallawy *et al*., 2005; Neuburger & Maschmeyer, 2006; Zarrilli *et al.*, 2009). Persistent neutropaenia with clinical deterioration also requires antibiotic administration up to two weeks (Legrand *et al*., 2011). The guidelines published by the National Comprehensive Cancer Network (NCCN) strongly recommends close monitoring of resistance patterns of pathogens, as well as consider prophylactic antimicrobial therapy only in patients at a high risk of developing an infection, and not for all patients, in order to prevent the emergence of resistance (NCCN: Prevention and Treatment of Cancer-Related Infections V1.2012). Furthermore, it is recommended to use carbapenems as a second-line of treatment, to prevent the emergence of resistance (Knapp & English, 2001). The strategy of antibiotic usage in neutropenic patients is that it should start immediately with the onset of febrile neutropaenia and first-line antibiotics should cover the most probable pathogens, based on the local epidemiology and patient history (Legrand *et al.*, 2011). Patient history is particularly important because the carriage of MDR strains may last several weeks or months, and may be latent until neutropaenia.

There is an urgent need for the development of novel antibacterial agents to combat the MDR organisms in the healthcare setting. However the pharmaceutical industry has reduced antibacterial drug development programs due to the low profit margins, and the current 'novel' antibacterials are merely a modification of already existing compounds (Rolston, 2009; Chopra *et al*. 2010). Doripenem, for example, is the latest carbapenem approved for clinical usage, but its activity is very similar to imipenem and meropenem (Rolston, 2009). Chopra *et al*., (2010) tested 1040 FDA approved drugs against a susceptible (ATCC 17978) and a resistant (BAA 1605) *A. baumannii* strains. Only 20 exhibited significant antimicrobial activity, and of these, only five compounds were active against the resistant strain, showing that even the susceptible strain (ATCC 17978) is naturally resistant to many classes of antibiotics.

1.6 Mechanisms of Antibiotic Resistance

A. baumannii is rapidly developing as a multi-drug resistance pathogen with the ability of acquiring and upregulating resistance mechanisms. All major resistance mechanisms reported in Gram-negative bacteria have been identified in *A. baumannii* such as modifications of target site, active efflux pumps, enzymatic deactivation of drugs and decreased influx (reviewed by Gordon & Wareham, 2010; Hawkey, 1998; McGowan, 2006). This has rendered all current major antibacterial agents such as penicillins, cephalosporins, aminoglycosides and quinolones as inefficient treatment options for *A. baumannii* infections, which can also partially be attributed to the intrinsic resistance that the organism harbours towards these classes of antibiotics (Dijkshoorn *et al.* 2007). Carbapenems have been the drug of last resort for treating *A. baumannii* infections, but the rise in resistance is very worrying.

Hancock (1998) suggested three classes of antibiotic resistance: Intrinsic resistance comprising mechanisms present in the species, irrespective of antibiotic exposure; acquired resistance comprising the induction of foreign resistance determinants but without change to the strain's genotype, and includes reversion to full susceptibility once the inducing conditions are removed; and lastly genetic resistance comprising the stable acquisition of novel resistance mechanisms as genetic material incorporated into the genome, which can occur by mutation of an existing gene or the acquisition of a foreign plasmid.

Recent research suggests that the observed increase in resistance can be attributed to the interplay of several resistance mechanisms: enzymatic and non-enzymatic, such as the decreased influx together with the expression of a resistance gene (Poirel & Nordmann, 2006; Poirel *et al.*, 2012).

1.6.1 Non-enzymatic mechanisms

1.6.1.1 The role of penicillin-binding proteins (PBPs) and outer-membrane proteins (OMP)

The role of PBPs in conferring antibiotic resistance in *A. baumannii* has been poorly investigated, but the reduced expression of PBPs has been reported to contribute to carbapenem resistance in isolates from Spain (Bou *et al.*, 2000; Giamarellou *et al.*, 2008). Another study found that the modification of PBPs was a source of imipenem resistance (Poirel & Nordmann, 2006).

Porin loss has been found to significantly contribute to resistance to carbapenems (reviewed by Dijkshoorn *et al.*, 2007; Giamarellou *et al.*, 2008). The loss of 22 and 33 kDa and OMPs together with the expression of an acquired β-lactamase resulted in carbapenem resistance (Bou *et al.*, 2000). CarO is an important OMP in the influx of carbapenems in *A. baumannii*,

and studies have shown that the disruption of carO by insertion elements contributes to carbapenem resistance in clinical isolates (Poirel & Nordmann, 2006).

1.6.1.2 The role of efflux pumps

Strain AYE, an epidemic multi-drug resistant *A. baumannii* strain was sequenced and the genome was found to encode several multi-drug efflux system (Fournier *et al*., 2006). AdeABC is best characterized efflux pump in *A. baumannii* (reviewed by Peleg *et al*. 2008; Giamarellou *et al*. 2008). It contains a three-component structure: AdeB forming the transmembrane component, AdeA forming the inner membrane fusion protein and AdeC forming the outer-membrane protein (reviewed by Peleg *et al*. 2008). It is also widely distributed in *A. baumannii* isolates and is chromosomally encoded (Dijkshoorn *et al*. 2007; Peleg *et al.* 2008). Interestingly, AdeABC acts against virtually all classes of antibiotics including carbapenems (Giamarellou *et al*. 2008; Nikaido 1998).

Tetracycline resistance is also mediated by transposon-mediated tetracycline-specific efflux pumps encoded by *tetA* and *tetB*, as well as ribosomal protection protein encoded by *tetM*, serving to protect the ribosome from the action of tetracycline (Giamarellou *et al*. 2008; Peleg *et al*. 2008; Perez *et al*. 2007).

1.6.1.3 The role of target site modifications

Target site modifications in *A. baumannii* seem to confer resistance to quinolones and aminoglycosides. Topoisomerase IV is the target of quinolones, and mutations at Ser80 and Glu84 of ParC subunit, combined with mutations at Ser83 of gyrA subunit contribute significantly to quinolone resistance (Dijkshoorn *et al.*, 2007; Vila *et al.*, 1997).

Aminoglycosides, on the other hand, bind to a highly conserved motif of 16s rRNA subunit, and methylation of *armA* prevents the antibiotic from binding to its target site, rendering the isolate resistant to aminoglycosides (Dijkshoorn *et al.*, 2007; Lee *et al.*, 2006; Peleg *et al*., 2008).

1.6.2 Enzymatic mechanisms of resistance

1.6.2.1 Aminoglycoside resistance

Aminoglycoside resistance is mediated by the AdeABC efflux pump as well as aminoglycoside modifying enzymes: phosphotransferases, acetyltransferases and nucleotidultransferases, with some strains containing multiple enzymes simultaneously belonging to the different classes (Perez *et al*., 2007; Seward *et al.*, 1998). Target site modification of *armA* is also responsible for aminoglycoside resistance, as described previously. Genes encoding aminoglycoside-modifying enzymes can be located on plasmids and transposons, and some have been identified on class-1 integrons (Nemec *et al.*, 2004). Seward *et al*. (1998) investigated 24 clinical *Acinetobacter* spp isolates from 11 countries and found that similar aminoglycoside modifying enzymes are present in unrelated isolates and the genes are not restricted to specific geographical areas. The location of these genes on mobile genetic elements such as plasmids and integrons contribute significantly to the wide dissemination of these genes around the world.

1.6.2.2 β-lactam resistance

β-lactam antibiotics are widely used in the hospital setting and comprise the penicillins, cephalosporins, monobactams and carbapenems. Their high efficacy and safety in clinical use

has increased the usage and consecutively resulted in emergence of resistance to β-lactam antibiotics (Livermore & Woodford, 2006). The main mechanism of resistance to β-lactam antibiotics is the production of β-lactamases, in addition to the non-enzymatic mechanisms previously mentioned such as target site alteration, changes in PBPs and efflux. There are four classes of β-lactamases described in *A. baumannii* (reviewed by Dijkshoorn *et al*., 2007).

The spread of β-lactamases correlates with the usage and development of β-lactam antibiotics. Benzylpenicillin, was the first β-lactam antibiotic but had poor penetration activity towards Gram-negative bacteria. The development of semi-synthetic penicillins was compromised by the spread of penicillinases among Gram-negative bacteria (Livermore & Woodford, 2006; Nordmann & Poirel, 2008). This was overcome by the development of third- and fourth- generation cephalosporins as well as β-lactamases inhibitors. The wide success of these compounds worldwide led to extensive usage in the clinical setting and subsequently resulted in emergence of resistance. Carbapenems, the last development of β-lactam antibiotics are now the 'drug of choice' for many serious and multi-drug resistant infections, but we are entering an era of resistance to carbapenems, no newly developed compounds, which will ultimately result in untreatable infections (Livermore & Woodford, 2006).

1.6.2.2.1 Class A β-lactamases

Class A β-lactamases are extended-spectrum β-lactamases (ESBLs) primarily responsible for resistance to broad-spectrum cephalosporins. The first class A β-lactamases was found in an *E. coli* isolate in 1965 and designated as bla_{TEM} (reviewed by Turner, 2005). The majority of ESBLs contain a serine at their active site and inhibited by clavulanic acid (Bush, 2001;

Bradford, 2001). They are more commonly identified in *Enterobacteriaceae*, but reports in *A. baumannii* are increasing around the world.

1.6.2.2.1.1 TEM-like β-lactamases

TEM-like enzymes were the first described and now the most commonly identified β-lactamases (reviewed by Bradford, 2001). The early enzymes, TEM-1 and TEM-2, were plasmid-mediated and had a spectrum limited to aminopenicillin resistance together with early-cephalosporin resistance (Naas *et al.*, 2008; Turner, 2005). The TEM-3 β-lactamase reported in 1987 was the first TEM-like enzyme to display ESBL properties (reviewed by Bradford, 2001).

TEM-92 and -166 are ESBLs identified in clinical *A. baumannii* isolates from Italy and the Netherlands (reviewed by Peleg *et al*. 2008). All Enzymes displaying ESBL phenotypes are TEM-1 and -2 derivatives, with mutations occurring in the active site, causing an enlargement thereby allowing the attack of the β-lactam ring (Livermore & Woodford, 2006; Poirel *et al*., 2012).

1.6.2.2.1.2 SHV-like β-lactamases

SHV-type β-lactamases are also narrow-spectrum predominantly present in *Enterobacteriaceae*, and can be both plasmid and chromosomally located (reviewed by Bradford, 2001; Poirel *et al*., 2012). There are slightly fewer derivatives of SHV-like variants than there are in the TEM enzymes (http://www.lahey.org/Studies/ last accessed 5 December 2012). SHV-like variants displaying ESBL phenotypes are SHV-2, SHV-5 and SHV-12 and have been reported worldwide (reviewed by Poirel *et al.*, 2012).

SHV-like enzymes are rarely reported in clinical *A. baumannii* isolates, but included reports from China and the Netherlands of *A. baumannii* isolates producing SHV-12 (reviewed by Perez *et al*. 2007).

1.6.2.2.1.3 CTX-M-like β-lactamases

CTX-M (CefoTaXiMease) enzymes were initially characterized in a clinical *E. coli* isolate in 1989 (Bauernfeind *et al*., 1990). CTX-M –type β-lactamases are extended-spectrum β-lactamases conferring resistance to penicillins and extended-spectrum cephalosporins, and they are plasmid mediated which largely facilitates the worldwide spread of these β-lactamases (reviewed by Poirel *et al.*, 2012). CTX-M enzymes can be classified into five clusters according to their amino acid sequences, with the main cluster implicated in *Enterobacteriaceae* clinical infections identified as the CTX-M-1 (Bonnet, 2004; Perez *et al.*, 2007; Poirel *et al.*, 2012). CTX-M-15, CTX-M-3 and CTX-M-1 enzymes are among the variants belonging to CTX-M-1 cluster and are prevalent in different regions of the world such as Europe, India and the Middle East (reviewed by Livermore & Woodford, 2006; Poirel *et al.*, 2012). However, although CTX-M enzymes are not commonly identified in *A. baumannii*, CTX-M enzymes belonging to cluster 2 have been reported in *A. baumannii*. CTX-M-2 carrying *A. baumannii* has been identified in a neurosurgery ward outbreak in Japan (Nagano *et al.*, 2004). Furthermore, reports from Bolivia and Pennsylvania have also detected *A. baumannii* producing CTX-M-2 and CTX-M-43, also a member of CTX-M-2 cluster (Adams-Haduch *et al*., 2008; Celenza *et al.*, 2006; Dijkshoorn *et al.*, 2007).

1.6.2.2.1.3 GES-like β-lactamases

GES-like enzymes are extended-spectrum β-lactamases which can be either plasmid or chromosomally located (Bonnin *et al.*, 2011; Poirel *et al*., 2012). *bla*$_{GES-1}$ was initially identified in *Klebsiella pneumoniae* in France (Poirel *et al.*, 2000). In *A. baumannii* GES-11, GES-12 and GES-14 have been identified in Belgium and France, where the patients had originated from Egypt, Palestine and Turkey (Bogaerts *et al.*, 2010; Bonnin *et al.*, 2011; Moubareck *et al.*, 2009). This indicates the worldwide dissemination of *bla*$_{GES}$ in different parts of the world. Interestingly, GES-14 possesses an amino acid change at position 170 (Gly170Ser) which contributes to extending the hydrolysis spectrum to include carbapenems (Bogaerts *et al.*, 2010; Poirel *et al*., 2012). GES-14 has been identified in France, Belgium and Turkey which raises a threat of the dissemination of this gene which could contribute to carbapenem resistance worldwide (Bogaerts *et al.*, 2010; Bonnin *et al.*, 2011).

1.6.2.2.1.4 VEB-like β-lactamases

VEB-like β-lactamases are distantly related to other ESBLs but confer high resistance to extended-spectrum cephalosporins in *Enterobacteriacea* and *Pseudomonas aeruginosa* worldwide, including Europe, Thailand and Kuwait (reviewed by Poirel *et al.*, 2012). VEB-1 was identified in *P. aeruginosa* in South East Asia as part of gene cassette located in a class 1 integron on the chromosome (Poirel *et al.*, 2003).

VEB-1 was identified in clinical isolates of *A. baumannii* involved in nosocomial outbreaks in France, Belgium and Argentina (Carbonne *et al.*, 2005; Naas *et al*., 2006; Pasterán *et al*., 2006). One strain in France was responsible for a nation-wide outbreak in 2003-2004 and belonged to Worldwide clone 1 (WW1) (Naas *et al.*, 2006; Poirel *et al.*, 2012). The outbreak

in Belgium was reported in hospitals located close to the French border and typing by pulsed-field gel electrophoresis and integron analysis revealed that the strains in Belgium were clonally related to those identified in the French epidemic (Naas *et al*., 2006).

1.6.2.2.1.4 PER-like β-lactamases

PER-like enzymes are widespread in *P. aeruginosa*, from which they get their name: *P. aeruginosa* extended resistance, and were initially described in 1993 from a Turkish patient in France (Poirel *et al.*, 2005). $bla_{\text{PER-1}}$ is commonly identified in *A. baumannii* isolates in Turkey, where it was reported to be present in 32% of ceftazidime resistant *A. baumannii* nosocomial infections (Kolayli *et al.*, 2005). Furthermore, PER-1 has been reported in France, Belgium, Japan and Russia (Bonnin *et al.*, 2011; Poirel *et al*., 2005).

PER-like variants are distinguished in two groups: point-mutant derivatives of PER-1 (PER-3, PER-4, PER-5 and PER-7), and PER-2 and PER-6 sharing 85% amino acid homology to PER-1.

PER-2 has been reported primarily in South America (Poirel *et al*., 2012), whereas PER-3 has been reported in Taiwan and France in *Aeromonas punctata* (Wu *et al*. 2011). PER-3 has also recently been identified in a clinical *A. baumannii* isolate in Egypt (Al-Hassan *et al.*, 2012). Localization of PER-3 can be either plasmid or chromosomal in *A. punctata,* but found to be chromosomally located in *A. baumannii* (Al-Hassan *et al*. 2012)

PER-7 contains four amino acid substitutions compared with PER-1 and has been detected in clinical *A. baumannii* isolates from the United Arab Emirates (UAE), France and recently Egypt (Bonnin *et al.*, 2011; Opazo *et al.*, 2012a; Al-Hassan *et al.*, 2012). The localization of PER-7 differed between the two geographical locations, with the French isolate harbouring a

chromosomal copy of *bla*$_{PER-7}$, whereas the isolate from UAE harboured a plasmid containing *bla*$_{PER-7}$; the genetic environment of both isolates were however identical (Bonnin *et al*., 2011; Opazo *et al.*, 2012).

1.6.2.2.1.4 Other class-A β-lactamases and secondary ESBLs

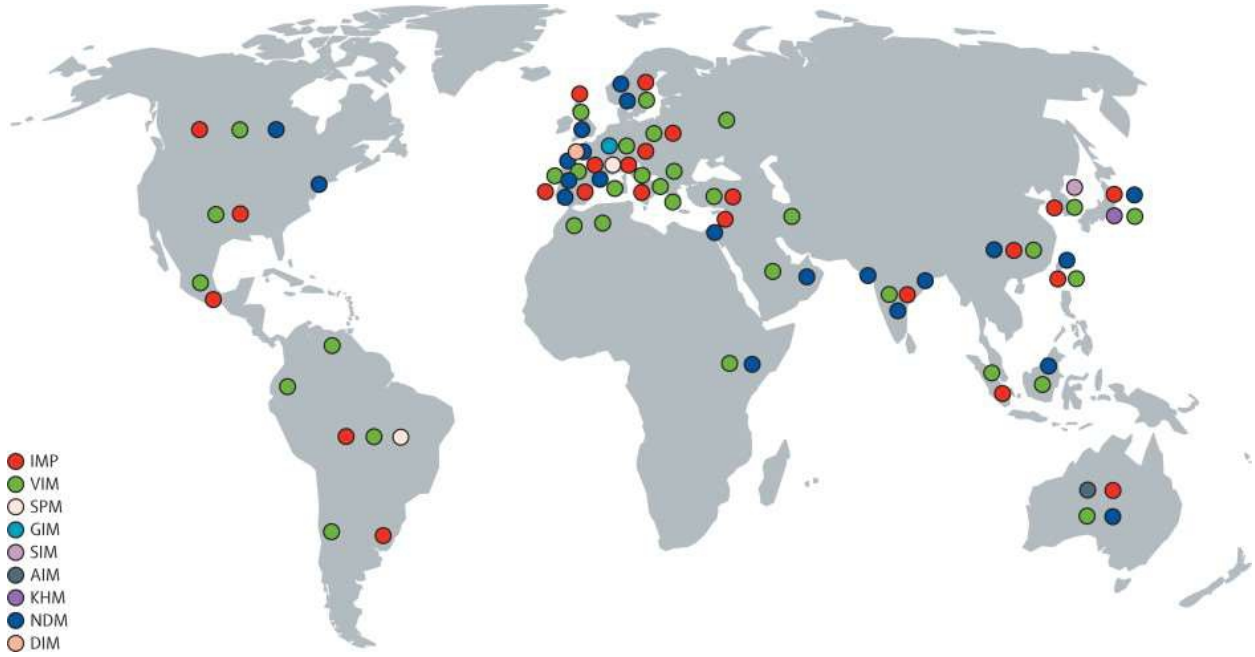
ESBLs rarely reported and which are confined to certain geographical areas are referred to as secondary ESBLs, as their clinical significance is not well studied yet and include: *bla*$_{BEL-like}$, *bla*$_{BES-like}$, *bla*$_{SFO-1}$, *bla*$_{TLA-like}$ and *bla*$_{PME-like}$ genes (reviewed by Poirel *et al*., 2012). They have been reported in Belgium, Brazil, Spain and Japan, Mexico and the USA, respectively, in different species such as *P. aeruginosa*, *S.marcescens* and *E. cloacae* but have not been characterized in *A. baumannii* (Poirel *et al*., 2012).

1.6.2.2.2 Class B β-lactamases

Class B β-lactamases are also known as metallo-β-lactamases (MBLs) due to the presence of zinc in the active site (Walsh, 2005). MBLs are particularly worrisome in the clinical setting due to their broad range, potent carbapenemase activity, and resistance to β-lactamase inhibitors. This is in addition to the frequent localization on mobile genetic elements, integrons and plasmids thereby facilitating spread (Cornaglia *et al.*, 2011; Walsh *et al*., 2005). MBLs were initially discovered in the 1960s, but only gained interest in the clinical setting after its identification in Japan and the dissemination of MBL genes in the clinical setting (Cornaglia *et al.*, 2011; Osano *et al*., 1994).

MBLs are classified in three structural classes according to the structure of their active site, where classes B1 and B3 contain two zinc ions and possess larger substrate specificity, in

contrast to class B2 with only one zinc ion (Cornaglia *et al*., 2011). Most important and clinically relevant acquired MBLs belong to class B1, such as IMP-type, VIM-type, SPM-type and NDM-type (Cornaglia *et al*., 2011; Walsh *et al*., 2005). As seen in figure 2, there is a worldwide distribution of MBLs. bla_{IMP} discovered in the late 1980s was localized in isolates from Asia, but later disseminated to other parts of the world. bla_{NDM} although being the most recently characterized MBL in 2009 in India, has also been found in several continents, including North America and Australia.



F**igure 2: The worldwide distribution of metallo-β-lactamases** (Taken from Cornaglia *et al.*, 2011).

MBLs are often present in *P. aeruginosa* isolates and are not predominant in *A. baumannii*, but their potential spread by horizontal gene transfer can lead to a serious problem in *A. baumannii* due to their carbapenemase activity (Perez *et al*., 2007; Cornaglia *et al*., 2011). Reports of MBL-producing *A. baumannii* have been from East-Asia, Taiwan, Korea, China,

Italy, and Greece, UAE, Israel, Germany and Egypt (Tsakris *et al*. 2006; Riccio *et al*. 2000; Cornaglia *et al*. 2011).

1.6.2.2.2.1 IMP-type β-lactamases

IMP-1 enzyme was first reported in Japan in 1988 in a clinical isolate of *P. aeruginosa* (Osano *et al*.,1994; Walsh, 2005). IMP-1 was limited to reports from Japan in different Gram-negative organisms such as *P. aeruginosa* and *K. pneumonia* (Riccio *et al*. 2000). A review by Walsh (2005) stated that Japan was a major reservoir for IMP-type MBLs including IMP-1, -2, -3, -6, -10 and -11, which have occurred in *Pseudomonas sp.*, *Acinetobacter sp.*, and *Enterobacteriaceae.* So far 38 IMP β-lactamases have been identified (http://www.lahey.org/Studies/ last accessed 5 December 2012).

In *A. baumannii* IMP-1, was characterized in isolates from Korea and Japan, and allelic variants including IMP-2, IMP-4, IMP-5, IMP-6 and IMP-11 have also been reported in clinical *A. baumannii* isolates from Italy, Japan, Hong Kong, Portugal and Brazil (Chu *et al*., 2001; Jones *et al*., 2004; Riccio *et al*., 2000). *IMP-like* genes have been found on class 1 integrons, which categorizes it as a transferable MBL (Walsh *et al*., 2005).

1.6.2.2.2.2 VIM-type β-lactamases

bla$_{\text{VIM-type}}$ MBL is also referred to as the 'European MBL' and is the second dominant group of MBL after IMP-type, with 34 known derivatives currently characterized (http://www.lahey.org/Studies/ last accessed 5 December 2012). VIM-1 was first reported in 1997 in a *P. aeruginosa* isolate from Italy, and characterized in 2006 in five *A. baumannii* isolates from Greece (Tsakris *et al*. 2006). We currently have reports of five VIM-type

variants in *A. baumannii*: VIM-1, -2, -3, -4, and -11 from Greece, Taiwan and Korea (Cornaglia *et al*., 2011; Lee *et al.,* 2008; Lin *et al.*, 2010). In the Greek study in 2006, the five *A. baumannii* isolates harbouring VIM-1 belonged to four distinct groups and were not related by PFGE, indicating the dissemination of the gene in different clones (Tsakris *et al*. 2006).

In a study performed in Taiwan on 260 non-duplicate Gram-negative organisms, 78 *A. baumannii* isolates were found to harbour three VIM-type genes: $bla_{\text{VIM-2}}$, $bla_{\text{VIM-3}}$ and $bla_{\text{VIM-11}}$ (Lee *et al.,* 2008). Furthermore, VIM-2 has also been identified in *Acinetobacter* 14TU in Germany (Tsakris *et al*. 2006).

1.6.2.2.2.3 SIM-type β-lactamases

$bla_{\text{SIM-1}}$ has been identified as a novel MBL in 2005 in Seoul, Korea (Lee *et al*., 2005). The study was performed to assess the prevalence of MBLs in *P. aeruginosa* and *A. baumannii* in a tertiary-care hospital in Seoul and identified seven *A. baumannii* isolates harbouring a novel MBL gene (Lee *et al.,* 2005). The gene was chromosomally located and carried on a class 1 integron, the PFGE results showed that the isolates belonged to two different clonal lineages (Lee *et al.*, 2005; Perez *et al.*, 2007). Furthermore, SIM-1 was found to share 69% similarity with IMP-12 and 64% similarity with IMP-9 (reviewed by Perez *et al*. 2007).

1.6.2.2.1.4 NDM-type β-lactamases

bla_{NDM} is the newest MBL characterized from a Swedish patient of Indian origin, who after a trip to New Delhi, India acquired a carbapenem-resistant *K. pneumonia* harbouring a novel MBL designated NDM-1; an *E. coli* isolate from the same patient's faeces was found to

harbour a 140kb plasmid containing *bla*$_{NDM,}$ highlighting the possibility of in-vitro conjugation (Yong *et al*., 2009). NDM-1 shares little identity with other MBLs, with most similarity to VIM-2 at 32.4% (Yong *et al.*, 2009). Further reports from India have shown a high prevalence of NDM-1-producing *Enterobacteriaceae* (Kaase *et al.*, 2011; Karthikeyan *et al.*, 2010).

A. baumannii isolates producing NDM-1 were found in India, China and Germany (Chen *et al.*, 2011; Karthikeyan *et al.*, 2010; Pfeifer *et al*., 2011). NDM-2, with one amino acid substitution at position 28 (proline to alanine), was identified in an *A. baumannii* isolate from a patient transferred to Germany after an accident and hospitalization in Egypt (Kaase *et al*., 2011). Reports from different regions of the Middle-East have emerged of NDM-2 producing *A. baumannii*, including Israel and the UAE (Espinal *et al*., 2011; Ghazawi *et al*., 2012).

Isolates from the UAE were isolated, 4 months apart, from a cancer patient previously treated in Egypt, Lebanon and the UAE, so the origin of the isolate is difficult to determine, but shows a local emergence of NDM-1 in the Middle East (Ghazawi *et al*., 2012). A study on the dissemination of NDM-1 producing *A. baumannii* in Europe showed that the five isolates from Germany, France, Slovenia and Switzerland belonged to 3 pulsotypes, which indicates a spread of NDM-producing clones in Europe (Bonnin *et al*., 2012).

1.6.2.2.3 Class C β-lactamases

AmpC enzymes are widespread in Gram-negative bacteria, and are naturally occurring class C β-lactamases in *A. baumannii*, with a serine at its active site, similar to class A β-lactamases (Jacoby, 2009). It is a chromosomally encoded cephalosporinase, also known as Acinetobacter-derived cephalospoinase (ADC), but its expression is very low and not

inducible except with the presence of an insertion sequence upstream (reviewed by Peleg *et al*., 2008). IS*Aba1* is commonly associated with the overexpression of $bla_{ADC,}$ providing a strong promoter resulting in high-level ceftazidime resistance (Corvec *et al*., 2003; Héritier *et al*., 2006). ADC enzymes are also penicillinase, which are able to hydrolyse narrow and broad spectrum cephalosporins, but not carbapenems or cefepime (Giamarellou *et al.* 2008; Perez *et al*. 2007). Observed resistance to cephalosporins can therefore be mediated by both class A and class C β-lactamases.

1.6.2.2.4 Class D β-lactamases

Class-D β-lactamases are also known as oxacillinases (OXA-type β-lactamases) and also contain serine at its active site, similarly to class A and C β-lactamases (Poirel *et al.*, 2010). OXA-type β-lactamases have carbapenem-hydrolysing activity (CHDL), which is alarming in the clinical setting (Walther-Rasmussen & Høiby, 2006). Their hydrolytic efficiency against carbapenems is nonetheless much lower than that observed for MBL (Poirel & Nordmann, 2006). The origin of OXA-type carbapenemases is unknown, but it is hypothesized that environmental bacteria carry these genes for protection against antibiotic-producing organisms in the soil, and the genes can then be transmitted via horizontal gene transfer to other bacteria (Walther-Rasmussen & Høiby, 2006). CHDL enzymes are widespread in *A. baumannii* being located on both the chromosome as well as acquired on foreign plasmids (Walther-Rasmussen & Høiby, 2006; Queenan & Bush, 2007). OXA-23, OXA-40, and OXA-58 are commonly identified acquired CHDL associated with carbapenem resistance in *A. baumannii*, whereas OXA-51-like enzymes are naturally occurring (reviewed by Peleg *et al*., 2008). The relatedness of these enzymes is seen in figure 3.

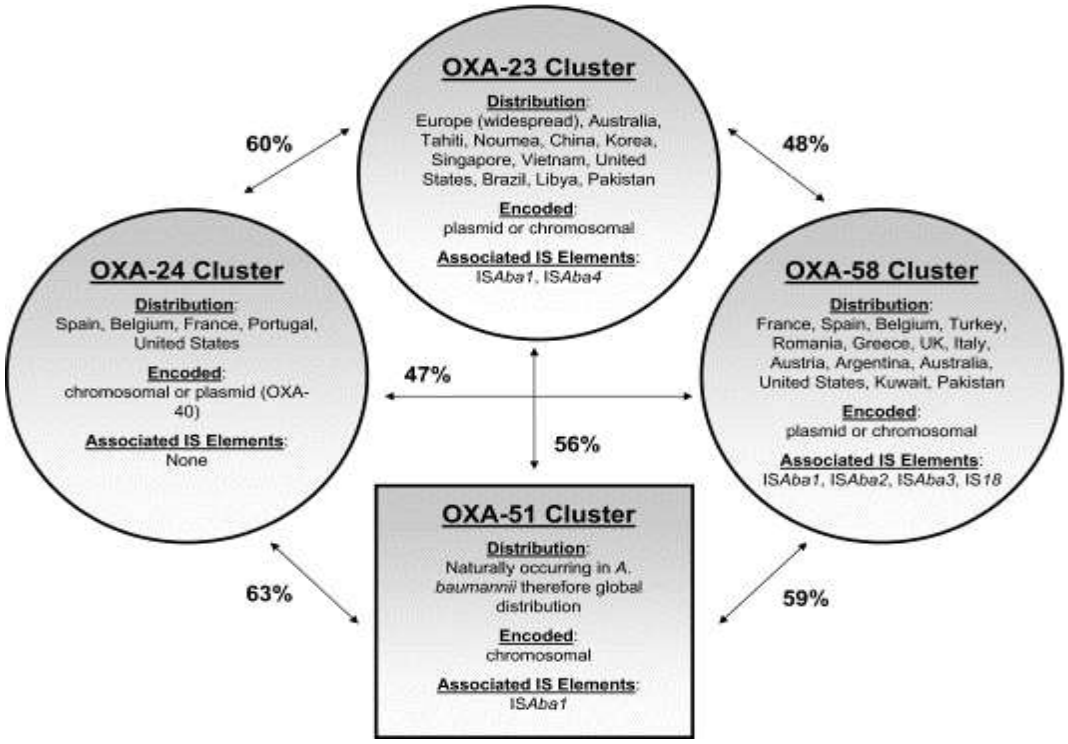


Figure 3: Distribution and relatedness of OXA-type carbapenemases in *A. baumannii* (Taken from Peleg *et al*., 2008)

1.6.2.2.4.1 OXA-23 Enzymes

OXA-23 was the first OXA-type carbapenemase identified in a strain from The Royal Infirmary of Edinburgh in 1985. The strain was resistant to all known antibiotics in addition to imipenem, which at the time, had not yet been used in the hospital (Paton *et al.*, 1993). The 'Acinetobacter resistant to imipenem', designated ARI-1 enzyme was demonstrated to be on a 40kb plasmid (Scaife *et al*., 1995), and was renamed OXA-23 after sequencing (Donald *et al*., 2000). It has been found in *A. baumannii* isolates causing outbreaks around the world (Paton *et al*., 1993; Queenan & Bush, 2007). The OXA-23 cluster, as seen in figure 3, comprises two other enzymes: OXA-27 and OXA-49 which differ to OXA-23 by two amino acid substitution each, and can be plasmid or chromosomally located (Walther-Rasmussen & Høiby, 2006; Queenan & Bush, 2007; reviewed by Peleg *et al*., 2008).

In an outbreak of carbapenem-resistant *A. baumannii* clones in London and Southeast England, two clones were identified circulating, both harbouring *bla*$_{\text{OXA-23}}$ as the resistance determinant (Coelho *et al.*, 2006). Military personnel from Iraq and Afghanistan were also infected and colonized with OXA-23 producing *A. baumannii* strains (Hujer *et al.*, 2006). Other outbreaks have been reported from Korea, China, Singapore, South America, Europe and the Middle East (Gur *et al.*, 2008; Jeon *et al.*, 2005; Mugnier *et al.*, 2008; Opazo *et al.*, 2012a; Opazo *et al.*, 2012b; reviewed by Peleg *et al.*, 2008; Zong *et al.*, 2008).

The genetic context of *bla*$_{\text{OXA-23}}$ has been investigated and revealed transposons Tn*2006*, Tn*2007* and Tn*2008* to be the structures harbouring this gene (Mugnier *et al.*, 2010). These transposons consist of either one or two copies of the insertion sequence (IS) IS*Aba1*. Recent reports have found IS*Aba10* inserted into the IS*Aba1* element upstream of the *bla*$_{\text{OXA-23}}$ gene, resulting in higher carbapenem MICs (Lee *et al.*, 2011). The role of IS elements with CHDL will be discussed in later sections.

The high incidence of *bla*$_{\text{OXA-23}}$ around the world has led researchers to investigate the progenitor of the gene in different *Acinetobacter sp* strains (Poirel *et al.*, 2008). Screening 50 *Acinetobacter sp* strains for *bla*$_{\text{OXA-23}}$ revealed that *Acinetobacter radioresistens*, a commensal organism, harboured a chromosomally located *bla*$_{\text{OXA-23,}}$ the gene which later disseminated to the opportunistic *A. baumannii*. *A. radioresistens* was however negative for IS elements which indicated that this organism does not serve as a reservoir to these IS elements involved in mobilization of the gene (Poirel *et al.*, 2008). The dissemination of *bla*$_{\text{OXA-23}}$ is uncertain, but has been proposed to have occurred by mobilization of the gene by a plasmid-mediated IS*Aba1* which in turn formed a transposon structure around *bla*$_{\text{OXA-23}}$, transposed and targeted a plasmid in the *A. radioresistens* progenitor, and finally this plasmid got transferred to *A. baumannii* via horizontal gene transfer or conjugation (Poirel *et al.*, 2008).

1.6.2.2.4.2 OXA-40 Enzymes

OXA-40, originally named OXA-24, was identified in a clinical *A. baumannii* isolate in 2000 from Spain. The isolate was resistant to all β-lactams, with MICs of 128mg/L and 256mg/L for imipenem and meropenem, respectively (Bou *et al.*, 2000). OXA-40 shares 60% similarity with OXA -23, but is not as widely distributed as OXA-23 (reviewed by Peleg *et al.*2008; Zarrilli *et al.* 2009) .

The OXA-40 cluster comprise OXA-25, OXA-26, OXA-40 and OXA-72, identified in Spain, Belgium, France and Thailand, respectively, sharing approximately 98% homology to OXA-40, (Afzal-Shah *et al.*, 2001; Poirel & Nordmann, 2006; Queenan & Bush, 2007; Walther-Rasmussen & Høiby, 2006). OXA-160 has also recently been identified in Pennsylvania as a new variant of OXA-40 enzyme (Tian *et al.*, 2011).

OXA-40 seems to be repeatedly involved in outbreaks in Spain and Portugal, but the gene has also been identified in other parts of the world, such as China, South Korea, Taiwan, USA, Iran and Bahrain (Poirel *et al.*, 2010). Figure 4 represents the distribution of the different OXA-type enzymes, and it is clear that reports of OXA-40 is less prevalent in carbapenem-resistant *A. bauamnnii* outbreaks than OXA-23 (Zarrilli *et al*. 2009).

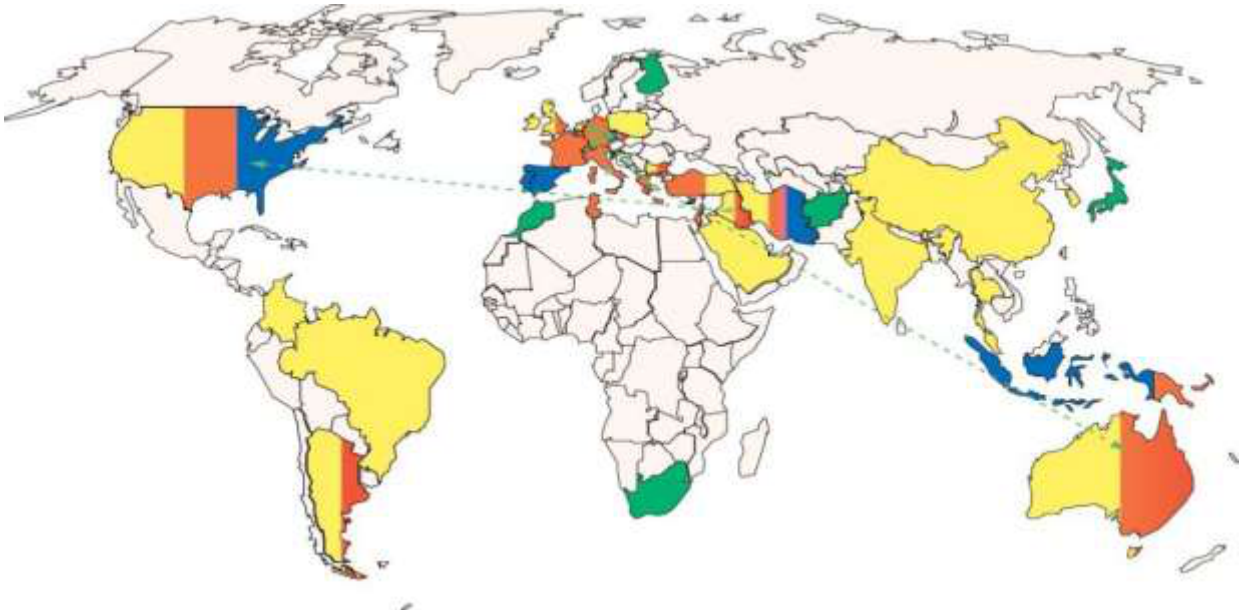


Figure 4: The geographical distribution of reports of carbapenem-resistant *A. baumannii* reports. OXA-23, OXA-40 and OXA-58 are indicated by the colours yellow, blue and red, respectively. Green indicates countries reporting carbapenem-resistant *A. baumannii* outbreaks in which the OXA-type enzyme has not been identified (Taken from Zarrilli *et al*. 2009)**.**

The localization of $bla_{\text{OXA-40}}$ can be both chromosomal or plasmid mediated and is not associated with any insertion sequences (reviewed by Peleg *et al*. 2008; Walther-Rasmussen & Høiby, 2006). In a large *A. baumannii-calcoaceticus* outbreak in Spain, plasmid mediated $bla_{\text{OXA-40/24}}$ was identified as the source of carbapenem resistance (Merino *et al*., 2010). Sequencing of the plasmid harbouring the gene revealed the presence of site-specific recombination sites: XerC/XerD flanking $bla_{\text{OXA-24}}$ suggesting its role in mobilization of the gene (Merino *et al*., 2010). The study, however, showed that there was no exchange of a common plasmid carrying $bla_{\text{OXA-40/24}}$ during the outbreak, but that the different plasmids exchanged the $bla_{\text{OXA-40/24}}$ gene, which was facilitated by the Xer recombination-site regions (Merino *et al*., 2010). This illustrates the different mechanisms of mobilization exhibited by the different OXA-type genes, and that the threat of carbapenem resistance is spreading to other *Acinetobacter sp.*, including environmental species.

The hydrolytic activity of this enzyme against carbapenems seems to be moderate, as well as weak activity against cephalosporins, indicating that they these enzymes are unable to combine ESBL and carbapenem-hydrolysing properties (Bou *et al*., 2000; Héritier *et al.*, 2005; Poirel *et al.*, 2010). The slow hydrolysis of carbapenems has led to speculation about the exact contribution of these enzymes in mediating carbapenem resistance in *A. baumannii*, but inactivation experiments of OXA-40 has rendered the isolate completely susceptible to carbapenems, thus endorsing the enzyme's role in carbapenem resistance (Héritier *et al.*, 2005; Poirel *et al.*, 2010).

1.6.2.2.4.3 OXA-51-like Enzymes

OXA-51-like enzymes are chromosomally located ubiquitous enzymes in *A. baumannii*. OXA-51 initially was identified in 2005 from genetically unrelated *A. baumannii* isolates from Argentina (Brown *et al.*, 2005), with several gene variants discovered in later years (Brown & Amyes, 2005; Evans *et al.*, 2007). OXA-51-like enzymes share 56% identity with OXA-23 cluster, and 63% with OXA-24 cluster (Peleg *et al*., 2008). Its penicillinase activity, together with effective hydrolysis of oxacillin confirmed it to be an OXA-type enzyme upon discovery (Brown *et al*., 2005)

$bla_{\text{OXA-51-like}}$ have weak carbapenem hydrolysing activity, and are only reported to contribute to carbapenem resistance if an insertion element is present upstream of the gene (Evans *et al*. ,2008). IS*Aba1*, IS*Aba9*, IS*Aba825* and recently IS*Aba2* have been associated with providing effective promoter sequences and contribute to carbapenem resistance (Lopes *et al.*, 2012; this thesis).

Evans *et al.* (2008) aimed to investigate the relationship among the OXA-51-like enzymes and the association of these enzymes with particular clonal lineages from worldwide sources

and a linkage map was constructed to reveal the relatedness of the enzymes. OXA-65 enzyme seems to form a central hub from which all other OXA-51-like enzymes radiate, which thereby suggests that OXA-65 is the ancestral enzyme (Evans *et al*., 2008). Furthermore, three families were formed around OXA-66, OXA-69 and OXA-98, indicating a close relationship among enzymes in the respective families, while the other enzymes appeared to form chains rather than groups (Evans *et al*., 2008). The cluster around OXA-66 formed the largest group, which correlated with their belonging to the Worldwide (formerly European) clone 2 (WW2), a highly successful lineage identified in different parts of the world (Evans *et al*., 2008). OXA-69 cluster is also commonly identified in Worldwide clone 1 (WW1), whereas OXA-71 has been associated with Worldwide clone 3 (WW3) (Merkier & Centrón, 2006; Evans *et al*., 2008). OXA-71 does not form a cluster, but a chain, and is closely related to OXA-64, now commonly reported in the Middle East (Mugnier *et al.*, 2009; Opazo *et al*., 2012a; personal results) The evolution of OXA-51-like enzymes seems to be an on-going process, with 81 enzymes currently identified, making the OXA-51-like one of the largest groups of β-lactamases (http://www.lahey.org/Studies/ last accessed 5 December 2012).

1.6.2.2.4.4 OXA-58 Enzymes

OXA-58 was identified from a carbapenem-resistant *A. baumannii* from Toulouse, France in 2005 (Poirel *et al.*, 2005). The enzyme effectively hydrolysed penicillins, oxacillins and imipenem, but not extended-spectrum cephalosporins, and the gene was identified on a 30 kb plasmid (Poirel *et al.*, 2005). OXA-58 shares 48% similarity to OXA-23 cluster, 47% to OXA-40 cluster and 59% to OXA-51 cluster (Peleg *et al*., 2008). Point mutant derivatives of OXA-58 are OXA-97 and OXA-96 identified in *A. baumannii* from Tunisia and Singapore, respectively (Poirel *et al.*, 2010). OXA-58 is increasingly reported in carbapenem-resistant *A.*

baumannii outbreaks from around the world, including Turkey, France, Argentina, Southeast Asia and the Middle East (Coelho *et al.*, 2006; Gur *et al.*, 2008; Peleg *et al.*, 2008; Poirel *et al.*, 2010). Figure 4 further illustrates the geographical distribution of OXA-58 outbreaks, which similarly to OXA-23 has a global distribution (Zarrilli *et al*., 2009).

Reports of OXA-58 have not been limited to *A. baumannii*, but have also included *A. junii*, *Acinetobacter pittii* (genospecies 3) and *Acinetobacter nosocomialis* (genospecies 13TU) (Evans *et al.*, 2010; Poirel *et al.*, 2010).

bla$_{\text{OXA-58}}$ is found to be plasmid mediated in most cases, and flanked by two copies of IS*Aba3*, in opposite orientation (Poirel *et al.*, 2005), however other promoter sequences have been reported associated with *bla*$_{\text{OXA-58}}$ such as IS*Aba2*, IS*Aba1* and IS*18* (reviewed by Peleg *et al.*, 2008; Poirel & Nordmann, 2006). Hybrid promoters, with IS*1006* and IS*1008* interrupting the upstream IS*Aba3*, have been reported in clinical *A. baumannii* and *A. calcoaceticus* isolates from Taiwan (Chen *et al.*, 2010; Chen *et al.*, 2008). This interruption appears to enhance transcription of *bla*$_{\text{OXA-58}}$ thus resulting in higher observed resistance. In addition to the importance of IS elements in *bla*$_{\text{OXA-58}}$ expression and resistance, another study showed that multiple copies of *bla*$_{\text{OXA-58}}$ on a plasmid resulted in higher MICs of carbapenem (Bertini *et al*., 2007). Furthermore, the level of resistance was still considerably higher in the original strains than in the transformants, which indicates additional resistance mechanisms in the clinical isolates (Bertini *et al*., 2007). It is therefore clear that multiple resistance mechanisms, such as alteration of porins and the expression of β-lactamases, act simultaneously in clinical isolates resulting in the observed resistance rates.

1.7 The genetic plasticity of *A. baumannii*

A. baumannii is a perfect example of genetic plasticity in bacteria, with its remarkable ability to acquire and up-regulate foreign genetic material, as well as the showing the role of different mobile genetic elements in mediating antibiotic resistance (Peleg *et al*., 2008; Vallenet *et al*., 2008)

A. baumannii strain AYE, a multidrug-resistant epidemic strain in France was studied by whole shotgun genome sequencing and revealed the presence of a 86-kb resistance island in which 45 resistance genes are clustered (Fournier *et al*., 2006). This genomic 'hot-spot' is an unstable genomic region which allows successive integration of resistance determinants from different bacterial strains, and the mosaic-like structure in strain AYE reveals the acquisition of DNA fragments from different bacterial species including *Pseudomonas*, *Salmonella* and *E. coli* (Fournier *et al*., 2006).

1.7.1 The role of plasmids in the genetic plasticity of *A. baumannii*

Plasmids act as important vehicles for the transmission and dissemination of various antibiotic resistance genes among different bacterial species through horizontal gene transfer (Fondi *et al*., 2010). The vast majority of resistance genes in *A. baumannii* have been shown to be localized on transferable plasmids (Poirel *et al.*, 2010;Walsh, 2005). Genes can effectively be mobilized on plasmids, transferred to other hosts and get disseminated in the hospital setting through horizontal gene transfer. Many outbreaks reported the presence of a circulating plasmid harbouring the resistance gene (Jeon *et al*., 2005; Gallego, 2010). Resistance genes located on plasmids can also get inserted into the bacterial genome by recombination. Plasmids are, therefore, important in facilitating genome rearrangement via

homologous recombination, resulting in the loss or acquisition of genes without altering the gene content of the bacterial chromosome (Fondi *et al*., 2010). This is facilitated by the abundance of transposable elements in plasmids. Most clinically significant carbapenem resistance in *A. baumannii* is associated with plasmid mediated acquisition of either MBLs or CHDL, which can furthermore integrate into the chromosome (Towner *et al*., 2011).

1.7.2 Integrons and their association with resistance genes

Integrons are capable of capturing, mobilizing and expressing resistance genes organised in gene cassettes (Poirel *et al.*, 2012). Several antibiotic resistance genes can be captured in the same gene cassette, thereby expressing resistance to several different antibiotic classes simultaneously (Weldhagen, 2004). Class 1 integrons are prevalent in clinical isolates and are particularly associated with class A β-lactamases (ESBLs), with VEB-1 being the first enzyme found to be encoded on a gene cassette in an integron (Naas *et al.*, 2006; Poirel *et al.*, 2012). The co-expression of resistance is seen in the frequent occurrence of aminoglycoside resistance genes together with a class-A β-lactamase in a gene cassette, meaning that the administration of one class of antibiotic, such as aminoglycoside, can select for ceftazidime resistance (Opazo *et al.*, 2012a; Weldhagen, 2004). Consequently, the role of integrons is to provide a gene capturing system useful in facing the challenge of multiple-antibiotic treatment regimens, thereby co-expressing simultaneous resistance (Mazel, 2006).

All integrons are composed of three elements necessary for gene capturing: an *intI* integrase gene, an *attI* primary recombination site and a promoter (P) (Mazel, 2006). Gene capturing and integration occurs with the intI-catalysed site-specific recombination between the attI site and the 59-base element located downstream of the resistance gene (Weldhagen, 2004).

Gene expression is mediated via the common promoter for all genes, rather than through individual promoters (Weldhagen, 2004)

The 3'-end of a class 1 integron-borne β-lactamases in *A. baumannii* commonly contain a *qacΔE1* and *sul1*, in addition to an aminoglycoside, rifampicin or chloramphinecol resistance genes (Opazo *et al.*, 2012a; Weldhagen, 2004). β-lactamases reported to be located in class 1 integrons include: SHV, GES, VEB and PER ESBL enzymes, IMP and VIM MBL enzymes (Opazo *et al.*, 2012a; Poirel *et al.*, 2000; Poirel *et al.*, 2012; Poirel *et al.*, 2005; Poirel *et al.*, 2003; Walsh, 2005; Weldhagen, 2004).

1.7.3 The role of insertion sequences as vehicles

The dissemination and spread of antibiotic resistance genes among bacteria is mediated by the localization of these genes on transposable elements, and the mobilization by insertion sequences (ISs) which are small and abundant transposable elements, capable of independent transposition in the genome (Mugnier *et al.*, 2009).

ISs are commonly associated with resistance genes belonging to class B, C and D, but less frequently class A, β-lactamases in *A. baumannii* (reviewed by Dijkshoorn *et al.* 2007; Jacoby, 2009). $bla_{\text{PER-1}}$ was found as part of a composite transposon, bracketed by IS*Pa12* and IS*Pa13,* and was not identified in a gene cassette or located inside a class 1 integron, unlike other PER-like enzymes (Poirel *et al*., 2005). IS*Aba1* has been, in *A. baumannii,* associated with several antibiotic resistance genes including $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51-like}}$ and bla_{AmpC} (Corvec *et al.*, 2003; Héritier *et al*., 2006; Mugnier *et al.*, 2009). The chromosomally located $bla_{\text{OXA-51-like}}$ gene has been reported to only confer resistance to carbapenems when IS*Aba1* is inserted upstream (Evans *et al.*, 2008), but more recently IS*Aba825* and IS*Aba2*

have also been associated with carbapenem resistance when located upstream of the *bla*$_{\text{OXA-51-like}}$ gene (Lopes *et al.*, 2012; this thesis). The genetic environment of *bla*$_{\text{OXA-23}}$ gene in *A. baumannii* has been identified to include either one or two copies of IS*Aba1*, with transposons Tn*2006*, Tn*2007*, Tn*2008* all identified as genetic structures harbouring the gene (Mugnier *et al.*, 2010). *bla*$_{\text{OXA-58}}$ are frequently associated with IS*Aba3* bracketing the gene in opposite directions (Poirel & Nordmann, 2006). For MBLs, NDM-type enzymes are also reported to be flanked by IS*Aba125*. Evidently, insertion sequences present upstream of the different resistance genes serves to not only mobilize the genes, but provide effective promoter sequences, hence facilitating their expression.

1.7.3.1 The role of common regions (CR)

Common regions (CRs) are present in class 1 integrons, often found close to the 3' conserved sequence, and resemble IS*91*-like family of insertion sequences (Toleman *et al.*, 2006a). IS*91*-like elements are distinguished from other classes of ISs in that they use rolling circle (RC) replication for transposition, allowing genetic rearrangements that other IS elements cannot easily perform (Toleman *et al.*, 2006b), hence proposing the term IS*CR*s.

The first IS*CR1* element was discovered in the early 1990s in class 1 integron as a 2154 bp DNA sequence, incorporating a putative gene with unknown function (orf*513*), located upstream of *sul1* genes (Toleman *et al*. 2006a).

IS*CR1* are associated with a number antibiotic resistance genes in different bacterial species, and act in the mobilization and dissemination of these genes by RC transposition process (Toleman *et al,*. 2006a). In *A. baumannii*, IS*CR1* has been associated with class A β-

lactamases: *bla*$_{PER-3}$ and *bla*$_{PER-7}$ (Opazo *et al*. 2012a; Toleman *et al*. 2006a; Al-Hassan *et al*. 2012).

Table 2 is a summary of the β-lactamases present in *A. baumannii*, the associated genetic and mobilization vehicles and their localization on plasmids or chromosomal.

Class of β-lactamase	Gene	Associated genetic structure	Localization
Class A β-lactamases	*bla*$_{\text{TEM-like}}$	Transposons: Tn*1*, Tn*2*, Tn*3*	Plasmid
	bla$_{\text{SHV-like}}$	Integrons, Insertion Sequence: IS*26*	Plasmid or chromosomal
	bla$_{\text{CTX-M-like}}$	Insertion Sequences, IS*CR1*	Plasmid
	bla$_{\text{VEB-like}}$	Integrons	Plasmid or chromosomal
	bla$_{\text{GES-like}}$	Integrons	Plasmid or chromosomal
	bla$_{\text{PER-like}}$	Integrons, IS*CR1*, Insertion Sequences: IS*4*.	Plasmid or chromosomal
Class B β-lactamases	*bla*$_{\text{IMP-like}}$	Integrons	Plasmid
	bla$_{\text{VIM-like}}$	Integrons	Plasmid
	bla$_{\text{SIM-like}}$	Integrons	Plasmid
	bla$_{\text{NDM}}$	Insertion Sequence: IS*Aba125*	Plasmid
Class C β-lactamases	*bla*$_{\text{AmpC}}$	Insertion Sequence: IS*Aba1*	Chromosomal
Class D β-lactamases	*bla*$_{\text{OXA-23-like}}$	Transposons: Tn*2006*, Tn*2007*, Tn*2008*. Insertion Sequence: IS*Aba1*	Plasmid
	bla$_{\text{OXA-40-like}}$	XerC/XerD-like recombination sites	Plasmid or chromosomal
	bla$_{\text{OXA-51-like}}$	Insertion Sequences: IS*Aba1*, IS*Aba2*, IS*Aba825*	Chromosomal
	bla$_{\text{OXA-58}}$	Insertion Sequences: IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*1006*, IS*1008*, IS*Aba825*	Plasmid

Table 2: Summary of β-lactamases in *A. baumannii*, their genetic environment and localization.

1.8 The issue of antibiotic resistance in developing countries

Antibiotic resistance is a global problem, but unfortunately some countries lack the appropriate surveillance and are unaware of the problem. The decline in antibiotic development and the increasing reports of resistance has led public health sectors to release reports of action plans to combat antibiotic resistance as well as proposing incentives for pharmaceutical investments in antibiotic research in the US and Europe (Spellberg *et al.*, 2008). This continuous surveillance of the epidemiology of infections and resistance rates, as well as the research undertaken in the developed world is not seen in the developing world. Limited data is available about the prevalence of hospital acquired infections, although the burden of infections is very high (Allegranzi *et al*., 2011).

A review of published papers about the prevalence of health-care associated infections in developing countries was carried out by Allegranzi *et al.* (2011) and they only identified 271 relevant publications from 1995-2008. The analysis revealed that the prevalence of health-care associated infections was much higher than data reported from Europe and the USA, reaching up to three-times as high. Reasons behind that include lack of infection control, inadequate hygiene, overcrowded wards, lack of surveillance and knowledge as well as no abidance of national and international guidelines (Allegranzi *et al*., 2011). Furthermore, Gram-negative pathogens represented the most common aetiology of nosocomial infections, but with limited reports of the associated resistance patterns (Allegranzi *et al*., 2011). Interestingly, *Acinetobacter sp*. was the second most frequent pathogen identified in ventilator-associated pneumonia and bloodstream infections.

Recently, some data and reports have been available concerning *A. baumannii* infections in the Middle East, and they have shown a surprising and interesting diversity. Heterogeneity was identified in *A. baumannii* isolates from Bahrain, with all three CHDL enzymes present

resulting in the observed resistance to carbapenems (Mugnier *et al.*, 2009). Similar heterogeneity is seen in *A. baumannii* isolates from a single centre in Cairo, in addition to the presence of several different $bla_{\text{OXA-51-like}}$ enzymes representing different clones (this thesis). The first report of a plasmid-mediate $bla_{\text{PER-7}}$ was found in a clinical isolate of *A. baumannii* from the United Arab Emirates (Opazo *et al.*, 2012a). $bla_{\text{NDM-2}}$ has been reported in *A. baumannii* isolates from in Egypt, Israel and the UAE (Espinal *et al.*, 2011; Kaase *et al.*, 2011; Ghazawi *et al*., 2012). American soldiers wounded in Iraq in 2003 were reported to have multi-drug resistant (MDR) *A. baumannii* (Hujer *et al.*, 2006). These results indicate two important observations: first of all, there is an increased awareness of the epidemiology of nosocomial infections in the Middle East, and second of all, the genome of isolates recovered from this region shows a large degree of diversity.

The lack of funding to undertake research in the developing world is one of the reasons behind the limited of reports available. Furthermore, there are no national surveillance systems, which in turn lead to poor and inaccurate data and un-standardized definitions. There is also limited communication between different healthcare facilities to alert of a certain outbreak. A large surveillance study was done in France and Belgium to contain the VEB-1 outbreak, where all hospitals were alerted managed to control the outbreak (Naas *et al.*, 2006). If similar measures are taken in the Middle East, it would greatly decrease the incidence of nosocomial infections and spread of resistance. There is an urgent need to create national and international surveillance systems across the Middle East.

International infectious disease surveillance has been conducted by the United States Department of Defence aiming to monitor the antimicrobial resistance by collaborating with civilian and military clinics, hospitals and universities worldwide, thereby creating a surveillance network (Meyer *et al*., 2011). Naval Medical Research Unit 3 (NAMRU-3) was created in 1946 and conducts research and surveillance to support military personnel

deployed to Africa, the Middle East, and Southwest Asia (http://www.med.navy.mil/sites/nmrc/Pages/namru3.htm last accessed 5 December 2012). Surveillance studies have shown that the most frequently identified Gram-negative HAI present in Egyptian hospitals are either ESBL-producing Enterobacteriaceae or *A. baumannii*. Furthermore, 37% of isolates are MDR with 15% being resistant to imipenem. *A. baumannii* presented approximately 15% among Gram-negative bacteria, being one the most important pathogens in hospital acquired infections in Egypt (Meyer *et al*., 2011). Interestingly, PFGE comparison of *A. baumannii* isolates from Egypt with isolates collected at a military treatment facility in the US showed high levels of genetic variability and diversity (Meyer *et al*., 2011).

1.9 Aims and objectives of this study

Given the emergence of *A. baumannii* as a major clinical pathogen with high degrees of genomic plasticity and diversity, we aimed to study *A. baumannii* from Egyptian cancer patients to determine the genetic diversity present in two centres in Cairo.

- Determine the prevalence of *A. baumannii* infections at The Children's Cancer Hospital as well as The National Cancer Institute.

- Study the clinical manifestations associated with *A. baumannii* infections in cancer patients.

- Study the epidemiological diversity present in *A. baumannii* from the two centres by using different methods: PFGE, MLST and sequencing of the intrinsic $bla_{\text{OXA-51-like}}$ gene to assess different methods of characterizing isolates of the same species

- Assess the presence of certain clones in the hospitals

- Investigate multi-drug resistance present in *A. baumannii* in Egyptian hospitals.

- Identify the diversity of resistance mechanisms and β-lactamase resistance in *A. baumannii* in Egyptian hospitals.

- Identify the role of different insertion sequences in the expression and regulation of OXA-type carbapenemases.

- Determine the genetic environments of antibiotic resistant genes.

Chapter 2: Materials and Methods

2.1 Collection of Bacterial Isolates

One hundred *A. baumannii* isolates were collected from March 2010 to June 2011 from paediatric cancer patients in The Children's Cancer Hospital 57357 (CCHE 57357) and the National Cancer Institute (NCI), both located in Cairo, Egypt. The isolates were initially identified phenotypically using standard microbiological techniques and by Phoenix and Vitek automated machines. The isolates were stored on -80° until further use.

2.2 Identification

Isolates were identified genotypically by amplification and sequencing of the intrinsic $bla_{\text{OXA-51-like}}$ gene. Further identification was performed by restriction analysis of the 16s-23s rRNA spacer sequence using *Alu*I and *Nde*II, as described earlier by Dolzani *et al*. (1995).

2.3 Clinical Data

The following information was collected from the patients' clinical records:

a. Date of Isolate
b. Location of the patient at time of infection
c. Disease
d. Patient demographics
e. Site of Infection
f. Outcome

2.4 Chemicals and Media

McConkey and Iso-Sensitest agars were used for subculture and growing of the isolates. They were purchased in powdered form from Sigma-Aldrich (Basingstoke, UK) and prepared according to the manufacturers' instructions.

Acinetobacter selective media was used to isolate *Acinetobacter spp.* in mixed cultures. Leeds Acinetobacter Medium (LAM) as previously described by Jawad *et al* (1994) was used in addition to Acinetobacter ChromAgar.

All chemicals and antibiotics utilised in this study were purchased by Sigma-Aldrich, unless otherwise stated.

2.5 Buffers

- 10X TAE

48.4g of Tris Base was added to 3.72g of EDTA (disodium salt) was dissolved in sterile distilled water, and 11.4 ml of Glacial acetic acid to a final volume of 1 litre. For gel running, 1X TAE buffer was used by diluting the solution 1:10.

- 10X TBE buffer

108g of Tris base, 55g of Boric Acid and 7.5 g of EDTA was dissolved in 1 litre of sterile distilled water. The pH was adjusted to 8.0 and the solution was sterilized. For Pulsed-Field Gel Electrophoresis, 0.5X TBE buffer was used for running.

- 1X TE buffer

1.21g of Tris base and 0.372g of EDTA (disodium salt) was dissolved in 1 litre of sterile distilled water. The pH was adjusted to 8.0 and the solution was sterilized.

- Lysis buffer

6.05g of Tris base, 18.61g EDTA (disodium salt) and 10g of N-Lauroyl Sarcosine was dissolved in 1 litre of sterile distilled water. The pH was adjusted to 8.0 and the solution was sterilized.

- Suspension buffer

12.11g of Tris base (0.1 M) was added to 37.22g of EDTA (disodium salt) and dissolved in 1 litre of sterile distilled water. The pH was adjusted to 8.0 and the solution was sterilized.

2.6 Minimum Inhibitory Concentration

Susceptibility testing of several classes of antibiotics including: Amikacin, Amoxicillin/Clavulanate, Aztreonam, Cefepime, Cefexime, Cefotaxime, Ceftazidime, Ceftriaxone, Ciprofloxacin, Imipenem, Meropenem, Pipracillin/Tazobactam, Trimethoprim/Sulfamethaxazole, Cefperazone/Sulbactam and Tobramycin is performed routinely in the microbiology laboratories at the NCI and CCH using two techniques: automated susceptibility and MIC using the Phoenix and Vitek machines, in addition to disk diffusion according to the CLSI guidelines v27 (http://www.microbiolab-bg.com/CLSI.pdf last accessed 14 December 2012). Susceptibility to Colisin was assessed by disk diffusion method according to the CLSI guidelines as mentioned above.

The minimum inhibitory concentration was subsequently performed for Imipenem, Meropenem and Ceftazidime according to the BSAC guidelines. A single colony from an

overnight culture was selected and grown in 5 mL of Iso-sensitest broth at 37^{o} in an orbital shaker at 180 rpm. The MIC was determined by agar double dilution technique according to the British Society of Antimicrobial Chemotherapy (BSAC) guidelines (British Society for Antimicrobial Chemotherapy, Version 6.1). The results were also interpreted according to BSAC guidelines (Andrews & Howe 2011). *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 were used as control strains.

2.7 Pulsed-Field Gel Electrophoresis (PFGE)

All isolates were typed by PFGE according to the procedure previously described by Seifert *et al*. (2005). The isolates were subcultured overnight on Iso-sensitest agar at 37^{o}. A loopful of bacteria was suspended in 3 mL of sterile cell suspension buffer. The final concentration of bacteria was adjusted to be between 1.7-2.0 nm at OD_{600}. Plugs were prepared by mixing 500 µl of this solution with 25 µl of Proteinase K (20 mg/L stock solution) and 500 µl of agarose (1.2% CHEF genomic agarose and 1% sodium dodecyl sulphate (SDS)) and dispensed into plug molds. The plugs were allowed to solidify for 10 minutes at 4^{o}, after which they were placed in 5 mL of cell lysis buffer and 25 µl of Proteinase K for 2 hours at 55^{o}. After lysis, the buffer was removed and the plugs were washed twice with distilled H_2O and 3 times with TE buffer. After the last wash, fresh TE buffer was used to store the plugs until further use.

For restriction, the plugs were cut with a scapel and transferred to the microcentrifuge tube containing the restriction mixture, which consisted of 100µl buffer with 30 U of *Apa*I. The plugs were restricted at 37^{o} overnight in a water bath and subsequently run on 1% pulsed-field-certified agarose gel (Bio-Rad, Hertfordshire, UK) in 0.5 X TBE buffer with initial

pulse of 5 and final pulse of 20 for 20 hours. The gels were stained with Gel-Red solution and seen using the Diversity Database software image capturing system.

2.8 Extraction of DNA

The isolates were subcultured at 37^{o} overnight, after which a loop-full of colonies was suspended in 200 µl of sterile distilled water. This mixture was boiled for 10-15 mins and then centrifuged at 6000g and the supernatant was used as DNA template.

2.9 Polymerase Chain Reaction (PCR) and Primers used

Genes were amplified by PCR in a total volume of 50 µl containing 5X Green GoTaq Flexi Buffer, 1.5 mM MgCl2, 800 µM PCR nucleotide mix, and 1.25 U GoTaq DNA polymerase (Promega, Southampton, UK). PCR conditions were as in table 3, unless stated otherwise:

	Temperature	**Time**	**Cycles**
Pre-Denaturation	94°	2 minutes	1
Denaturation	94°	40 seconds	30
Annealing	Variable	50 seconds	
Extension	72°	1 minutes	
Final Extension	72°	5 minutes	1
Cooling	4°		

Table 3: PCR conditions used in this study

Primers used were diluted to 100 and 25 pmol as stock and working concentrations, respectively. Primers listed below were either designed for the study using Biotools software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi last accessed 10 August 2012) or from previously published papers.

PCR products were analysed on 1% agarose, stained with GelRed and viewed using the Diversity Database software image capturing system (Bio-Rad, Hemel Hempstead, UK).

For sequencing, PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and sequenced in both directions, with 3.2 pmol primer concentration.

- PCR for Identification of *A. baumannii* isolates

Primers used were previously reported by Dolzani *et al*. (1995) to confirm the identification of *A. baumannii* isolates. After amplification, products were digested with *Alu*I and *Nde*II for more specific characterization of species. The fragments specific for *A. baumannii* produced by *Alu*I restriction enzymewere: 50, 125, 135, 165, 177 and 330 bp. Fragments produced by *Nde*II were: 50, 110,145, 330 and 360 bp.

Primer Name	Sequence 5'-3'	Fragment size	Annealing Temperature
16s-23s rRNA F	TTG TAC ACA CCG CCC GTC A	975 bp	55°
16s-23s rRNA R	GGT ACT TAG ATG TTT CAG TTC		

Table 4: Primers used for identification

- Amplification and Characterization of *bla*$_{\text{OXA-51-like}}$ gene

Primers used to amplify the intrinsic *bla*$_{\text{OXA-51-like}}$ gene were previously reported by Heritier *et al.* (2005). To further characterize the gene, products were purified and sequenced.

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
Oxa69-A	CTA ATA ATT GAT CTA CTC AAG	975 bp	48°
Oxa69-B	CCA GTG GAT GGA TGG ATA GAT TAT C		

Table 5: Primers used for amplification and sequencing of *bla*$_{\text{OXA-51-like}}$

A larger fragment size was seen for isolates harbouring an insertion sequence upstream of the *bla*$_{\text{OXA-51-like}}$ gene. The upstream region of *bla*$_{\text{OXA-51-like}}$ was subsequently amplified and sequenced in both directions by using primers FxOxaF and FxOxaR, previously described by Lopes (2012), as well as mixing primers FxOxaF and Oxa69-B, and preABprom and OXA-51R (Heritier *et al* 2006*;* Woodford *et al.* 2006).

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
FxOxaF	GAT ACC AGA CCT GGC AAC AT	889 bp	57°
FxOxaR	GCA CGA GCA AGA TCA TTA CC		
preABprom	GAC CTG CAA AGA AGC GCT GC	variable	52°

Table 6: Primers used for amplification and sequencing of the upstream region of *bla*$_{\text{OXA-51-like}}$ genes

- Detection of Acquired OXA Carbapenemases

Multiplex PCR, previously described by Woodford *et al*. (2006) was used to screen the isolates for OXA carbapenemases.

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
OXA-23F	GAT CGG ATT GGA GAA CCA GA	501 bp	52°
OXA-23R	ATT TCT GAC CGC ATT TCC AT		
OXA-24F	GGT TAG TTG GCC CCC TTA AA	246 bp	
OXA-24R	AGT TGA GCG AAA AGG GGA TT		
OXA-58F	AAG TAT TGG GGC TTG TGC TG	599 bp	
OXA-58R	CCC CTC TGC GCT CTA CAT AC		
OXA-51F	TAA TGC TTT GAT CGG CCT TG	501 bp	
OXA-51R	TGG ATT GCA CTT CAT CTT GG		

Table 7: Primers used for multiplex PCR of acquired OXA carbapenemases

- Amplification and sequencing of *bla*$_{\text{OXA-23}}$

The full *bla*$_{\text{OXA-23}}$ gene was amplified and sequenced using primers by Afzal-Shah *et al.* (2001) and Heritier *et al.* (2007) for isolates harbouring the gene by multiplex PCR described above.

The presence of IS*Aba1* upstream of *bla*$_{\text{OXA-23}}$ was studied using a combination of primers IS*Aba1*-F, reported by Corvec *et al.* (2007) and OXA-23R, reported by Woodford *et al.*(2006).

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
bla$_{OXA-23}$ F	GAT GTG TCA TAG TAT TCG TCG	1026 bp	56°
bla$_{OXA-23}$ R	TCA CAA CAA CTA AAA GCA CTG		
OXA-23A	GGA ATT CCA TGA ATA AAT ATT TTA CTT GC	840 bp	55°
OXA-23B	CGG GAT CCC GTT AAA TAA TAT TCA GGT C		
ISAba1A	GTGCTTTGCGCTCATCATGC	1100 bp	55°

Table 8: Primers used for amplification and sequencing of *bla*$_{OXA-23}$

- Amplification and sequencing of *bla*$_{OXA-58}$

The full *bla*$_{OXA-58}$ and associated upstream environment was amplified and sequenced by mixing ISAba3-F and SM2 primers described by Poirel *et al*. (2006) with OXA-58R used in multiplex PCR method. The downstream environment was amplified and sequenced by mixing OXA-58F primers, used in multiplex PCR, with ISAba3-R, reported by Poirel *et al*. (2006).

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
SM2	AAG TGT CTA TAT TCT CAC C	Variable	56°
ISAba3-F	CAA TCA AAT GTC CAA CCT GC	900 bp	55°
ISAba3-R	CGT TTA CCC CAA ACA TAA GC		

Table 9: Primers used for amplification and sequencing of *bla*$_{OXA-58}$

- Amplification and sequencing of *bla*$_{\text{OXA-24/40}}$

The full *bla*$_{\text{OXA-24/40}}$ was amplified and sequenced using primers described by Jeon *et al*. (2005).

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
OXA-24FF	ATG AAA AAA TTT ATA CTT CCT ATA TTC AGC	825 bp	56°
OXA-24RR	TTA AAT GAT TCC AAG ATT TTC TAG C		

Table 10: Primers used for amplification and sequencing of *bla*$_{\text{OXA-40/24}}$

- Detection of *bla*$_{\text{ADC}}$

PCR was performed to amplify the *bla*$_{\text{ADC}}$ gene in *A. baumannii* isolates using primers previously reported by Ruiz *et al*. (2007).

For larger fragment sizes produced by isolates harbouring an insertion sequence upstream of *bla*$_{\text{ADC}}$, primers ISADC1 and 2 were used for the detection of IS*Aba1* upstream of *bla*$_{\text{ADC}}$ according to Ruiz *et al.* (2007).

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
ADC1	CCG CGA CAG CAG GTG GAT A	420 bp	52°
ADC2	TCG GCT GAT TTT CTT GGT T		
ISADC1	GTT GCA CTT GGT CGA ATG AAA A	751 bp	52°
ISADC2	ACG TCG CGA GTT GAA GTA AGT T		

Table 11: Primers used for amplification and sequencing of *bla*$_{\text{ADC}}$

- PCR for the detection of Extended Spectrum Beta-lactamases (ESBLs)

Isolates resistant to Ceftazidime were screened for the presence of ESBLs by amplification and sequencing of *bla*$_{TEM}$ (Weill *et al*. (2004), *bla*$_{VEB}$ (Lopes *et al*, 2012), *bla*$_{SHV}$ (Findlay *et* al. 2012), *bla*$_{PER}$ (This thesis), *bla*$_{GES}$ (Lopes, 2012) *bla*$_{CTX-M}$ (Woodford *et al.*, 2005).

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
TEM- F	GTT GCA CTT GGT CGA ATG AAA A	1080 bp	57°
TEM-R	ACG TCG CGA GTT GAA GTA AGT T		
SHV-F	CGC CGG GTT ATT CTT ATT TG	1069 bp	57°
SHV-R	CCA CGT TTA TGG CGT TAC CT		
VEB-F	ATT TCC CGA TGC AAA GCG T	360 bp	57°
VEB-R	CCA ACA GCG ATG AAC AAA CT		
PER-F	CCT GAC GAT CTG GAA CCT TT	715 bp	56°
PER-R	GCA ACC TGC GCA ATG ATA GC		
GES-F	ATG CGC TTC ATT CAC GCA C	840bp	55°
GES-R	AAC TCA TCC TGA GCA CGG AC		
CTX-M-F	AAA AAT CAC TGC GCC AGT TC	415bp	52°
CTX-M-R	AGC TTA TTC ATC GCC ACG TT		

Table 12: Primers used for detection of ESBLs

- PCR for detecting the genetic environment of *bla*$_{PER-like}$ genes

The genetic environment of *bla*$_{PER-like}$ genes (See Results section), was analysed by mixing primers of Int1 by Opazo *et al.* (2012a), 3'CS variable region (This study), Sul1, orf*513* and AYE-3396, by Opazo *et al*. (2012a), IS*Pa12* and IS*Pa13* by Poirel *et al*. (2005). Products were purified and sequenced accordingly to obtain full sequences.

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
Int1 F	GTT GCA CTT GGT CGA ATG AAA A	600 bp	52°
Int1 R			
ORF513-F	TCA AAG AGA CGA CTC TGT GAT GGA T	400 bp	55°
ORF-513-R	TGA CTC TTA TCC AAC GCT TTG GC		
5'CS	GGC ATC CAA GCA GCA AG	Variable	55°
3'CS	AAG CAG ACT TGA CCT GA		
AYE-3396 R	GGG TTT CCG AGA AGG TGA TT	Variable	56°
ISPa12 B	GAT CTC GCT TTA CAT TTA CC	Variable	55°
ISPa12A	ACA ATC GCT GAT ATA CAT CG		
PER ext1	CCT GCA ACA GTA CCT GCT TG		
ISPa13B	GGT ATC CAC CAC ATA TGG GC		55°
ISPa13A	TAA CCA TAT GCA CTC AAC GG		

Table 13: Primers used for amplification and sequencing of *bla*$_{\text{PER-like}}$ genetic environment

- Amplification for Metallo-β-lactamase genes (MBLs)

PCR was performed to detect MBLs with primers designed for this study.

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
IMP-F	GGA ATA GAG TGG CTT AAY TCT C	188 bp	53°
IMP-R	CCA AAC YAC TAS GTT ATC T		
VIM-F	GAT GGT GTT TGG TCG CAT A	390 bp	53°
VIM-R	CGA ATG CGC AGC ACC AG		
SIM-F	TAC AAG GGA TTC GGC ATC G	570 bp	56°
SIM -R	TAA TGG CCT GTT CCC ATG TG		
NDM -F	GGG CCG TAT GAG TGA TTG	650 bp	52°
NDM-R	GCA CAC TTC CTA TCT CGA C		

Table 14: Primers used to detect MBL

2.10 Multi-locus Sequence Typing

Ten Isolates, representative of each *bla*$_{\text{OXA-51-like}}$ type from the two hospitals, were further typed by Multi-locus sequence typing according to the scheme published by Barutal *et al*. (2005). Briefly, DNA was prepared as previously described, and used as template for the PCR of the 7 house-keeping genes: *gltA*, *rpoD, gpi, gyrB*, *gdhB*, *recA* and *cpn60*. Products were subsequently purified and sequenced in both directions, analysed, and isolates were assigned to sequence types using tools on the A. baumannii MLST webpage http://pubmlst.org/abaumannii/.

The PCR conditions were as follows:

	Temperature	Time	Cycles
Pre-Denaturation	94°	2 minutes	1
Denaturation	94°	1 minute	30
Annealing	Variable	1 minute	
Extension	72°	2 minutes	
Final Extension	72°	2 minutes	1
Cooling	4°		

Table 15: PCR conditions for MLST

Primers were designed by Bartual *et al*. (2005) but modifications were made to the annealing temperatures as shown below.

Primer Name	Sequence 5'-3'	Fragment Size	Annealing Temperature
Citrato F1	AAT TTA CAG TGG CAC ATT AGG TCC C	722 bp	55°
Citrato R12	GCA GAG ATA CCA GCA GAG ATA CAC G		
APRU F	TGT AAA ACG ACG GCC AGT GCN GGR TCY TTY TCY TGR CA	909 bp	45°
UP1E R	CAG GAA ACA GCT ATG ACC AYG SNG GNG GNA ART TYR A		
GDHB 1F	GCT ACT TTT ATG CAA CAG AGC C	775 bp	59°
GDHB 775R	GTT GAG TTG GCG TAT GTT GTG C		
RA1	CCT GAA TCT TCY GGT AAA AC	425 bp	55°
RA2	GTT TCT GGG CTG CCA AAC ATT AC		
CPN 3F2	ACT GTA CTT GCT CAA GC	479 bp	59°
CPN R2	TTC AGC GAT GAT AAG AAG TGG		
GPI F1	AAT ACC GTG GTG CTA CGG G	508 bp	55°
GPI R1	AAC TTG ATT TTC AGG AGC		
70F RPOD	ACG ACT GAC CCG GTA CGC ATG TAY ATG MGN GAR ATC GCN CAN CT	492 bp	58°
70R RPOD	ATA GAA ATA ACC AGA CGT AAG TTN GCY TCN ACC ATY TGY TTY TT		

Table 16: Primers used for amplification and sequencing for MLST

Sequencing of *gyrB*, *gdhB* and *rpoD* required different primers sequences than those used for sequencing. Primers are listed below.

Primer Name	Sequence 5'-3'	Gene
M13 [−21]	TGT AAA ACG ACG GCC AGT	*gyrB*
M13 F	CAG GAA ACA GCT ATG ACC	
GDH SEC F	ACC ACA TGC TTT GTT ATG	*gdhB*
GDH SEC R	GTT GGC GTA TGT TGT GC	
70FS	ACG ACT GAC CCG GTA CGC ATG TA	*rpoD*
70RS	ATA GAA ATA ACC AGA CGT AAG TT	

Table 17: Primers used for sequencing in MLST

2.10.1 e-BURST analysis

Evolutionary relationships and clonal complexes within the isolates were investigated by eBURST analysis, using the software on the eBURST website (http://eburst.mlst.net/v3/enter_data/single/ last accessed 16 December 2012)

2.11 S1 nuclease

S1 nuclease was used to detect and size plasmids. Plugs were digested with 10 units of s1 nuclease at 37° for 45 minutes, and subsequently run on PFGE. Gels were stained with Gel-Red and viewed by the gel-doc system.

2.12 Plasmid curing

Plasmid curing was performed on strains harbouring bla_{PER-7} and bla_{PER-3} to detect if the plasmid harbouring the resistance gene was lost. The strains were serially subcultured for 10

consecutive days and incubated at elevated temperatures. Ceftazidime resistance was measured by disk-diffusion method after curing and compared with the zone exhibited by the strain before curing. S1 nuclease was also performed to confirm the loss of plasmid.

2.13 Conjugation

Conjugation was attempted with the isolate harbouring $bla_{\text{PER-3}}$ (See results). *E. coli* J62.2 was used as a recipient. The donor and recipient were cultured overnight in IST broth, after which they were mixed in 1:4 ratio. Cells were collected by centrifugation and inoculated in 30μL of saline. 5 μL of this suspension was plated on Nutrient Agar plates containing Rifampicin (16 mg/L) and Ceftazidime (512 mg/L).

2.14 Phenotypic detection of MBL

All isolates were screened for MBL production by using Imipenem-EDTA Disk Method as described by Yong *et al*. (2003). Isolates were Nutrient Agar and disks containing Imipenem (30μg) and EDTA disks (5 and 20mM) were placed 10 mm apart. Plates were incubated at 37° for 18-20 hours and MBL production was confirmed when the imipenem zone was expanded by EDTA.

2.15 RNA extraction and Reverse Transcription

RNA was extracted using the RiboPure Bacteria Kit (Applied Biosystms). RNA was reverse transcribed to cDNA by incubation at 45°C/1 hr, followed by PCR gene amplification of $bla_{\text{OXA-51-like}}$ according to the manufacturer's instructions (Access RT-PCR System Kit, Promega).

2.16 Sequence Interpretation : BLAST and multalin

Sequencing was performed by Sanger method using an ABI 373A DNA sequencer (PE Applied Biosystems, Warrington, UK). All sequences were interpreted by BLAST (http://blast.ncbi.nlm.nih.gov/ last accessed 15 October 2012) and sequence alignment of DNA or amino acids were performed using Multalin tool (http://www.toulouse.inra.fr/multalin.html Last accessed 15 October 2012).

Chapter 3: Results

3.1 Collection of bacterial isolates

Cancer patients are prone to several opportunistic infections due to their immune-suppresive state. It was therefore expected to isolate mixed organisms from patients during their febrile neutropaenia episode. Work undergone in Cairo included storing all non-fermenting Gram-negative organisms, identifying them phenotypically as well as using automated machines such as Pheonix and Vitek.

Several cultures obtained contained mixed organisms: Gram-positive and Gram-negative, or mixed Gram-negative. Therefore purification of the cultures was done in these cases. A total of 100 isolates were identified as non-fermenting Gram-negative *Acinetobacter* spp. *A. baumannii* was identified in 46 isolates, while the remaining 54 were identified as *Acineotbacter* sp. or *A. lwoffii*. Phenotypic and automated identification is not nearly as accurate as molecular identification. Further genotypic identification by restriction analysis of the 16s-23s rRNA spacer sequences using *Alu*I and *Nde*II as well as amplification and sequencing of the $bla_{\text{OXA-51-like}}$ gene, served to identify the *Acinetobacter sp* as well as confirm the *A. baumannii* isolates. Due to the large number of patients in the hospitals, many samples are processed during a regular working day, which is why the microbiology laboratories at CCH and the NCI rely on automated machines for identification and susceptibility testing of the organisms. Due to the large similarities shared by isolates in the *A. baumannii-A. calcoaceticus* complex, automated machines poorly distinguish between them. Molecular methods, on the other hand, are specifically tailored for accurate identification.

Thirteen *Acinetobacter sp.* isolates were identified as *A. baumannii* genotypically. Additionally, 10 isolates identified as *A. baumannii* by the automated machines, were found to be non-baumannii genotypically, but part of the *A. baumannii-A. calcoaceticus* complex. The remaining 21 isolates were identified as *A. baumannii* both by the automated machines and genotypically. A total of thirty-four non-duplicate *A. baumannii* isolates were used in the study. Some patients had several *A. baumannii* cultures from different sites within a small time period, therefore cultures taken within one week from the same patient were considered in the same episode, and only one culture was chosen for further studies.

3.2 Automated and phenotypic susceptibility testing

Routine susceptibility testing in the centres in Cairo is done by disk diffusion according to the CLSI guidelines. Susceptibility is tested furthermore using the automated machines as stated earlier.

For the 100 *Acinetobacter sp* identified, overall resistance (MIC >4mg/L or zone diameter >17mm) was 64% to Amikacin, 67% to Amoxillin/Clavulanate, 58 % to Aztreonam, 65% to Cefepime, 67% to Cefexime, 68% to Cefotaxime, 62% to Ceftazidime, 73% to Ceftriaxone, 57% to Ciprofloxacin, 53% to Imipenem, 57% to Meropenem, 58% to Pipracillin/Tazobactam, 66% to Trimethoprim/Sulfamethaxazole, 47% to Cefoperazone/Sulbactam and 26% to Tobramycin. All isolates were susceptible to Colistin by disk diffusion (disk content 10 mg, zone diameter ≥11 mm).

3.3 Clinical data associated with isolates

Of the 34 *A. baumannii* isolates, 25 were recovered from patients in the Children's Cancer Hospital (CCH) and the remaining nine were from patients hospitalized in the National Cancer Institute (NCI).

Clinical data was unavailable for some patients hospitalized at the NCI. Only data obtained from the microbiology report could be incorporated into the analysis.

The age of patients ranged from 8 months to 25 years, with the median age of 5 years.

All patients had an underlying malignancy, with the majority of patients (n=20) having haematological disease (leukaemia or lymphoma) and seven patients with solid tumours.

As seen in table 18, twenty-four isolates were recovered from blood or CVP-related cultures, accounting for 70% of the total. Six isolates were recovered from respiratory and wound/pus samples and four GIT and urine samples accounted for 18% and 12%, respectively. In a report by El-Mehallawy *et al.* (2005), the majority of isolates were recovered from blood or CVP-related sources from Egyptian cancer patients, thereby indicating a problem in Egyptian hospitals.

ISOLATE NUMBER	HOSPITAL	DATE OF SAMPLE	SITE OF ISOLATE	AGE (Years)	SEX	DIAGNOSIS	WARD	OUTCOME
4248	CCHE	15/03/2010	CVP tip	9	F	Non-hogkins Lymphoma	IP - 5C	Alive
4343	CCHE	17/03/2010	CVP Blood	>1	F	Neuroblastoma	IP - 3B	Alive
4842	CCHE	23/03/2010	CVP tip	2	F	Rhabdomyosarcoma	ICU	Dead
7947	CCHE	17/05/2010	Wound	5	M	Medulloblastoma	Stepdown 2014	Dead
9930	CCHE	15/06/2010	Blood	1.9	M	Acute Myeloid Leukemia	Stepdown 2016	Dead
10262	CCHE	19/06/2010	CVP	2	M	Non-hogkins Lymphoma	ICU	Alive
12435	CCHE	23/07/2010	Blood	4	M	Non-hogkins Lymphoma	ICU	Dead
14298	CCHE	22/08/2010	Cath tip	5	F	Hodgkin's Disease	ICU	Alive
14611	CCHE	27/08/2010	CVP Blood	1.2	M	Rhabdomyosarcoma	ICU	Dead
15094	CCHE	05/09/2010	Urine	9	F	Osteosarcoma	IP - 4B	Alive
15324	CCHE	09/09/2010	Cath tip	2	F	Acute Myeloid Leukemia	IP - 3A	Alive
21174	CCHE	09/12/2010	Blood	4	M	NHL - Burkitt's Lymphoma	IP - 3A	Alive
21382	CCHE	13/12/2010	CVP Culture	4	F	Acute Lymphoblastic Leukemia	ICU	Dead
22055	CCHE	25/12/2010	CVP Blood	15	F	Acute Lymphoblastic Leukemia	IP - 3C	Alive
161	CCHE	03/01/2011	Sputum	6	F	Hodgkin's Disease	MSC - PULM	Alive
634	CCHE	11/01/2011	Cath tip	3	F	Non-hogkins Lymphoma	ICU	Dead
1447	CCHE	24/01/2011	CVP Culture	16	F	Acute Myeloid Leukemia	ICU	Alive
1780	CCHE	31/01/2011	Stool	8	F	Non-hogkins Lymphoma	Stepdown 2015	Dead
1750	CCHE	31/01/2011	BAL	11	F	Hodgkin's Disease	ICU	Dead
2106	CCHE	08/02/2011	Blood	8	M	Acute Myeloid Leukemia	Stepdown 2012	Dead
2632	CCHE	20/02/2011	Stool	1.6	F	Intraventricular ATRT	IP - 5A	Alive
2625	CCHE	20/02/2011	Urine	14	M	Ewing Sarcoma	IP - 4C	Alive
7052	CCHE	5/7/2011	BAL	>1	M	Astrocytoma	ICU	Alive
8357	CCHE	5/29/2011	CVP Blood	>1	M	Neuroblastoma	DSCH	Alive
8768	CCHE	6/4/2011	Blood	>1	M	JMML	IP - 4B	Alive
Abd	NCI	02/09/2010	Ear swab	14	M	Acute Myeloid Leukemia	5th floor - room 5	Alive
Bas	NCI	04/09/2010	Ear swab	12	F	Acute Myeloid Leukemia	5th floor - room 2	Dead
P391 - Ahm	NCI	14/09/2010	Blood	2.8	M	Acute Lymphoblastic Leukemia	5th floor	Dead
Shay	NCI	11/10/2010	Blood	2	F	Non-hogkins Lymphoma		Alive
461 - Seif	NCI	15/12/2010	Blood		M		7th floor	
Sam	NCI	15/12/2010	Blood	25	M	Acute Lymphoblastic Leukemia	7th floor	Dead
P38 - Ysf	NCI	04/01/2011	Blood		M		5th floor	
P49 - Ham	NCI	05/01/2011	Blood		M		5th floor	
P67 - Azak	NCI	09/01/2011	Blood	5	M		OP	

Table 18: Clinical and demographical data for isolates. CVP: Central Venous Port, JMML: Juvenile myelomonocytic leukaemia, MSC-PULM: Pulmonary clinic. OP: Out-patient ward, IP: In-patient ward

The location of patients at time of infection varied considerably. CCH has three floors for in-patient (IP) admissions, two floors for ICU, bone-marrow transplantation unit and the surgical department, and one floor for out-patient admissions and clinics. The NCI however has two floors for paediatric oncology admissions in addition to outpatient clinics floor, whereas the rest of the building is for adult malignancies and surgery. The location of patients

at the time of infection varied considerably in CCH. The majority of isolates (n=14) were recovered from patients in the ICU, four isolates from patients hospitalized in the 3rd IP floor, three isolates from patients at the 4th IP floor, two isolates from 5th IP floor, and two isolates from outpatient clinics. As for the NCI the distribution of isolates were more clustered with the majority (n=5) being recovered from patients hospitalized in paediatric oncology 5th floor, one isolate from the adult ward (7th floor) and one isolate from the outpatient clinics.

The patients were monitored during their Febrile Neutropenia episode during which they had *A. baumannii* infection. Mortality was only recorded if the patient died within the Febrile Neutropenia episode and outcome was hence recorded two weeks after the end of the *A. baumannii* infection and before the onset of the next chemotherapy treatment. In terms of patient outcome, 38% (n=13) died after the *A. baumannii* infection. For patients at CCH, they were hospitalised in the ICU and most suffered from an underlying haematological malignancy. The reason for this is the chemotherapy regimen for haematological malignancy is more immuno-ablative, which makes the patients more prone to opportunistic infections. It is debatable whether death in those patients can be attributed to the *A. baumannii* infections or not, as the existence of co-morbidity can significantly affect the outcome of the patient.

No specific distribution was seen for the sex of patients with *A. baumannii* infection.

3.3.1 Patient Origin and Governorates

The two centres from which the isolates were obtained are tertiary referral hospitals, so patients come from different governorates in Egypt. Data was only obtainable from patients in CCH, and identified 11 different governorates. Table 19 shows the number of patients originating from each governorate. As seen in figure 5, there was a large geographical

distribution of patient origin. The relevance of this diversity will be explained in later sections.

GOVERNORATE	NUMBER OF PATIENTS
Alexandria	2
Bani Sweif	2
Cairo	3
El-Dakahleya	4
El-Fayoum	2
El-Monofeya	1
El-Sharqeya	2
Giza	4
Kafr El Sheikh	1
Qina	2
Sohag	1

Table 19: Distribution of patients originating from different governorates in Egypt.

Figure 5: Map of Egypt showing the different governorates

(6th of October is now part of the Giza governorate)

3.4 Diversity in *bla*$_{\text{OXA-51-like}}$ gene

Amplification and sequencing of *bla*$_{\text{OXA-51-like}}$ gene revealed a large diversity among the strains with 8 difference genes identified. This is peculiar given the relatively short time of study (1.5 years) and that the strains were obtained from only two hospitals.

Genes identified were *bla*$_{\text{OXA64}}$**,** *bla*$_{\text{OXA65,}}$ *bla*$_{\text{OXA66,}}$ *bla*$_{\text{OXA69,}}$ *bla*$_{\text{OXA71,}}$ *bla*$_{\text{OXA78,}}$ *bla*$_{\text{OXA94,}}$ *bla*$_{\text{OXA89/100.}}$ Details are listed in table 20. In each case below the sequence is compared against the recognised standard sequence for each *bla*$_{\text{OXA}}$ gene.

ISOLATE NUMBER	HOSPITAL	DATE OF SAMPLE	SITE OF ISOLATE	*bla* OXA-51-like GENE	WARD
4343	CCHE	17/03/2010	CVP Blood	64	IP - 3B
7947	CCHE	17/05/2010	Wound and Sputum	64	Stepdown 2014
12435	CCHE	23/07/2010	Blood	64	ICU
14298	CCHE	22/08/2010	Cath tip	64	ICU
8357	CCHE	5/29/2011	CVP Blood	64	DSCH
4248	CCHE	15/03/2010	CVP tip	65	IP - 5C
4842	CCHE	23/03/2010	CVP tip	65	ICU
9930	CCHE	15/06/2010	Blood and CVP	65	Stepdown 2016
10262	CCHE	19/06/2010	CVP	65	ICU
15094	CCHE	05/09/2010	Urine	65	IP - 4B
15324	CCHE	09/09/2010	Cath tip	65	IP - 3A
1780	CCHE	31/01/2011	Stool	65	Stepdown 2015
1750	CCHE	31/01/2011	BAL	65	ICU
2106	CCHE	08/02/2011	Blood	65	Stepdown 2012
2632	CCHE	20/02/2011	Stool	65	IP - 5A
2625	CCHE	20/02/2011	Urine	65	IP - 4C
8768	CCHE	6/4/2011	Blood	65	IP - 4B
14611	CCHE	27/08/2010	CVP Blood	66	ICU
21382	CCHE	13/12/2010	CVP Culture	66	ICU
7052	CCHE	5/7/2011	BAL	66	ICU
634	CCHE	11/01/2011	Cath tip	69	ICU
1447	CCHE	24/01/2011	CVP Culture	69	ICU
161	CCHE	03/01/2011	Sputum	71	MSC - PULM
21174	CCHE	09/12/2010	Blood	78	IP - 3A
22055	CCHE	25/12/2010	CVP Blood	100	IP - 3C
Bas	NCI	04/09/2010	Ear swab	64	5th floor - room 2
P67 - Azak	NCI	09/01/2011	Blood	64	OP
Sam	NCI	15/12/2010	Blood	64	7th floor
P391 - Ahm	NCI	14/09/2010	Blood	65	5th floor
461 - Seif	NCI	15/12/2010	Blood	65	7th floor
Abd	NCI	02/09/2010	Ear swab	66	5th floor - room 5
Shay	NCI	11/10/2010	Blood	71	5th floor
P38 - Ysf	NCI	04/01/2011	Blood	94	5th floor
P49 - Ham	NCI	05/01/2011	Blood	94	5th floor

Table 20: Isolates harbouring the respective *bla*$_{\text{OXA-51-like}}$ genes, with date, site and location of patients.

3.4.1 *bla*$_{\text{OXA-64}}$

bla$_{\text{OXA-64,}}$ now common in the Middle East, was found in both CCH and NCI in a total of eight isolate. No correlation was found among isolates harbouring *bla*$_{\text{OXA-64,}}$ as they were

isolated in different months and anatomical sites. Interestingly, isolates from CCH harbouring *bla*$_{\text{OXA-64}}$ were all from patients in the ICU.

Silent mutations in the nucleotide sequences are seen in position 43 (A to C) for 7947 and P67-AZak, in addition to position 44 (T to C) for 7947, as seen in figure 6.

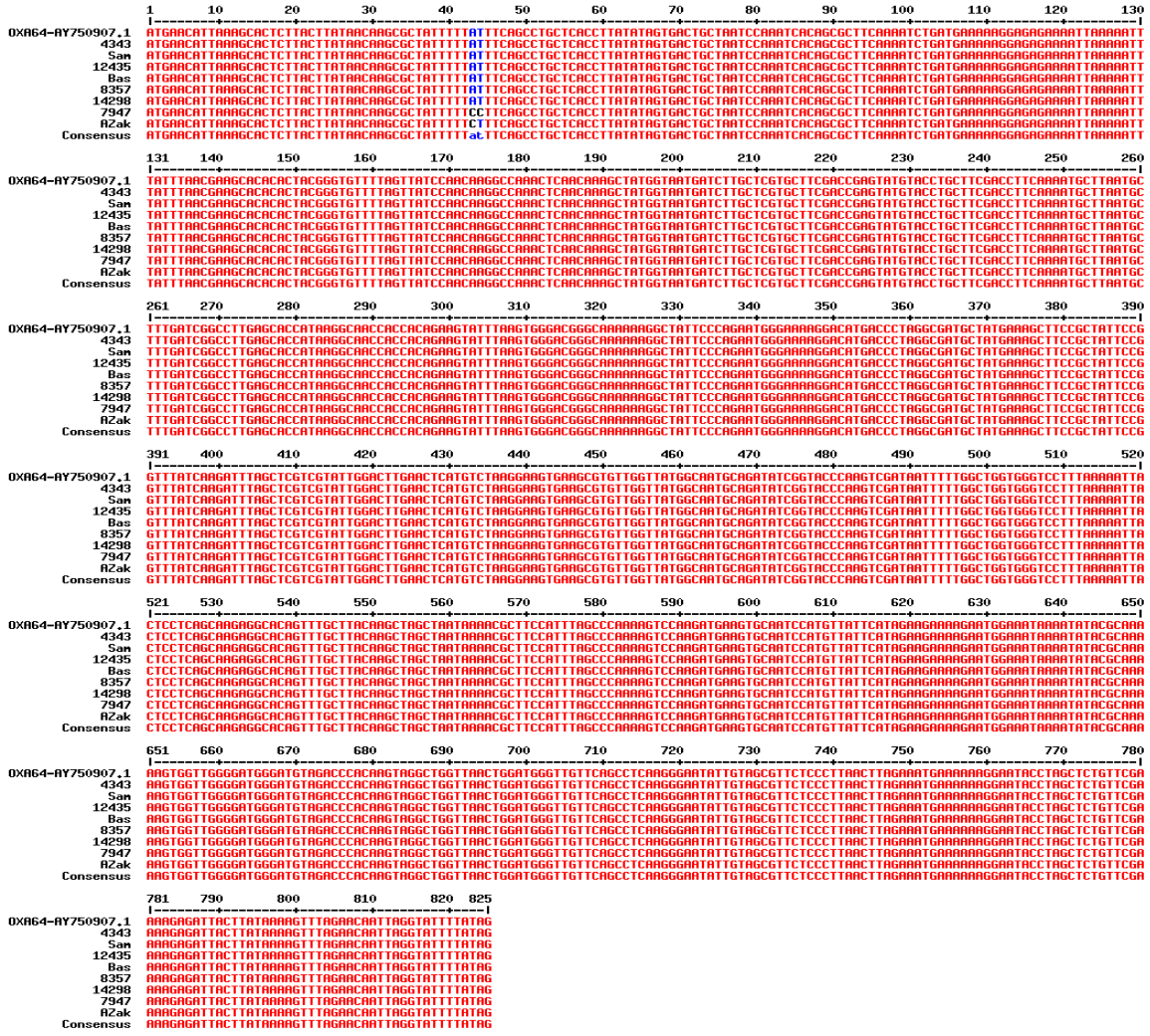


Figure 6: Nucleotide alignment for *bla*$_{\text{OXA-64}}$

Isolates harbouring *bla*$_{\text{OXA-64}}$ were typed by PFGE and their profiles compared as seen in figure 7. No epidemiological linkage can be seen between the profiles, as all isolates are

<80% similar in their patterns. Maximum similarity is seen at 60% for isolates 12435 and 8357 from CCH in addition to 679-BAS and P67-AZak from the NCI.

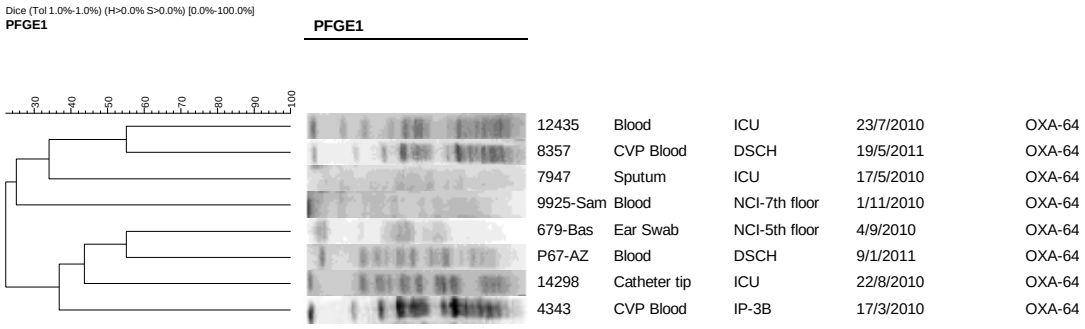


Figure 7: PFGE profiles for *bla*$_{\text{OXA-64}}$ isolates

3.4.2 *bla*$_{\text{OXA-65}}$

bla$_{\text{OXA-65}}$ was the most prevalent gene identified, found in 14 isolates from both hospitals. As seen in table 20, isolates harbouring *bla*$_{\text{OXA-65}}$ gene were distributed in several wards of both hospitals and they were isolated in different months. Only 3 isolates, 1750, 1780 and 2106 were from an ICU outbreak in early 2011, and harboured the *bla*$_{\text{OXA-65}}$ gene.

Three silent mutations were present in all isolates harbouring *bla*$_{\text{OXA-65}}$ except isolate P361-AH from the NCI (Figure 8). These mutations were present at positions 90 (T to C), 636 (C to T) and 663 (A to G). Additionally, isolates 4842 and 8768 harboured silent mutations at positions 801 (T to A) and 808 (A to G), respectively.

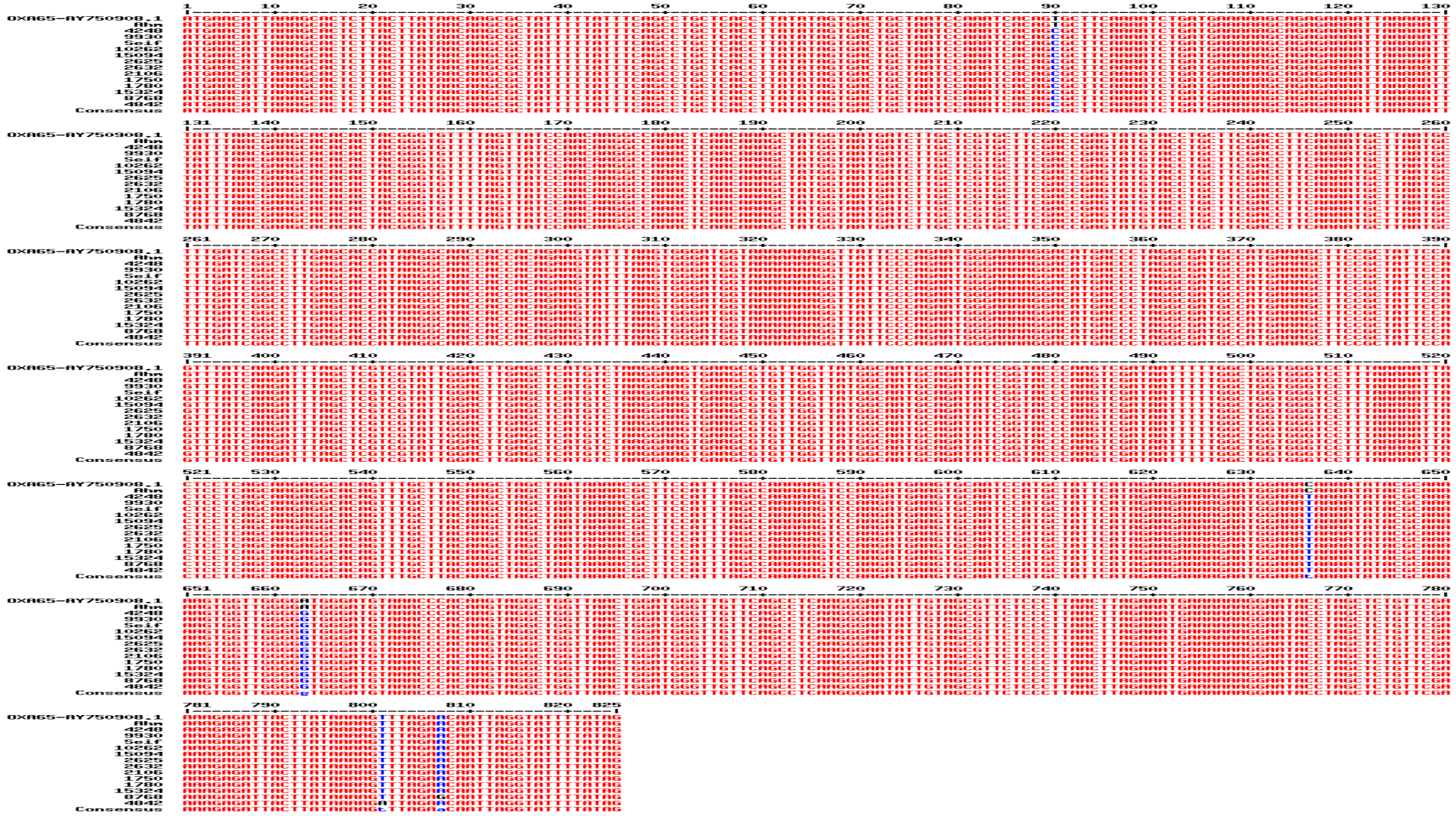


Figure 8: Nucleotide alignment for isolates harbouring *bla*$_{\text{OXA-65}}$

PFGE patterns of the isolates were compared as seen in figure 9. Isolates 461-SF and 4842 seem to be clonally related, in addition to isolates 10262 and P361-AH, as they share >80% similarity in their pattern. The isolates were from different hospitals and detected in different months. Additionally, isolates 1750 and 1780, which were from the ICU outbreak, also have similar patterns.

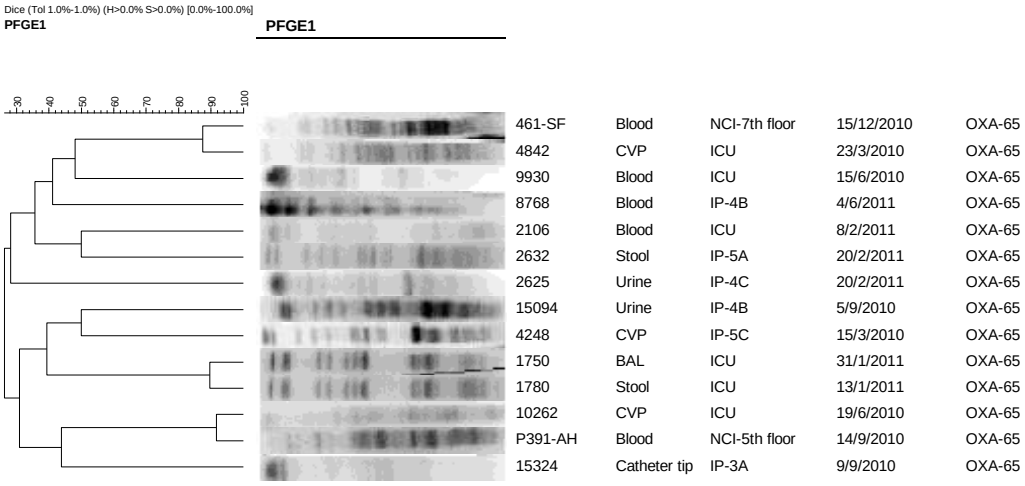


Figure 9: PFGE profiles for *bla*$_{\text{OXA-65}}$ isolates

3.4.3 *bla*$_{\text{OXA-66}}$

Three isolates harbouring *bla*$_{\text{OXA-66}}$ were recovered from patients at the CCH, all of which were hospitalized at the ICU but in different months. One isolate was also recovered from a patient in the NCI.

Fifteen silent mutations were identified in isolates harbouring *bla*$_{\text{OXA-66}}$ (figure 10). These mutations were at positions 90 (T to C), 107 (A to T), 108 (C to A), 171 (A to T), 177 (T to C),

309 (A to G), 318 (G to T), 319 (G to A), 328 (C to T), 349 (A to G), 369 (T to C), 390 (G to A), 511 (C to T), 636 (C to T), 801 (T to C).

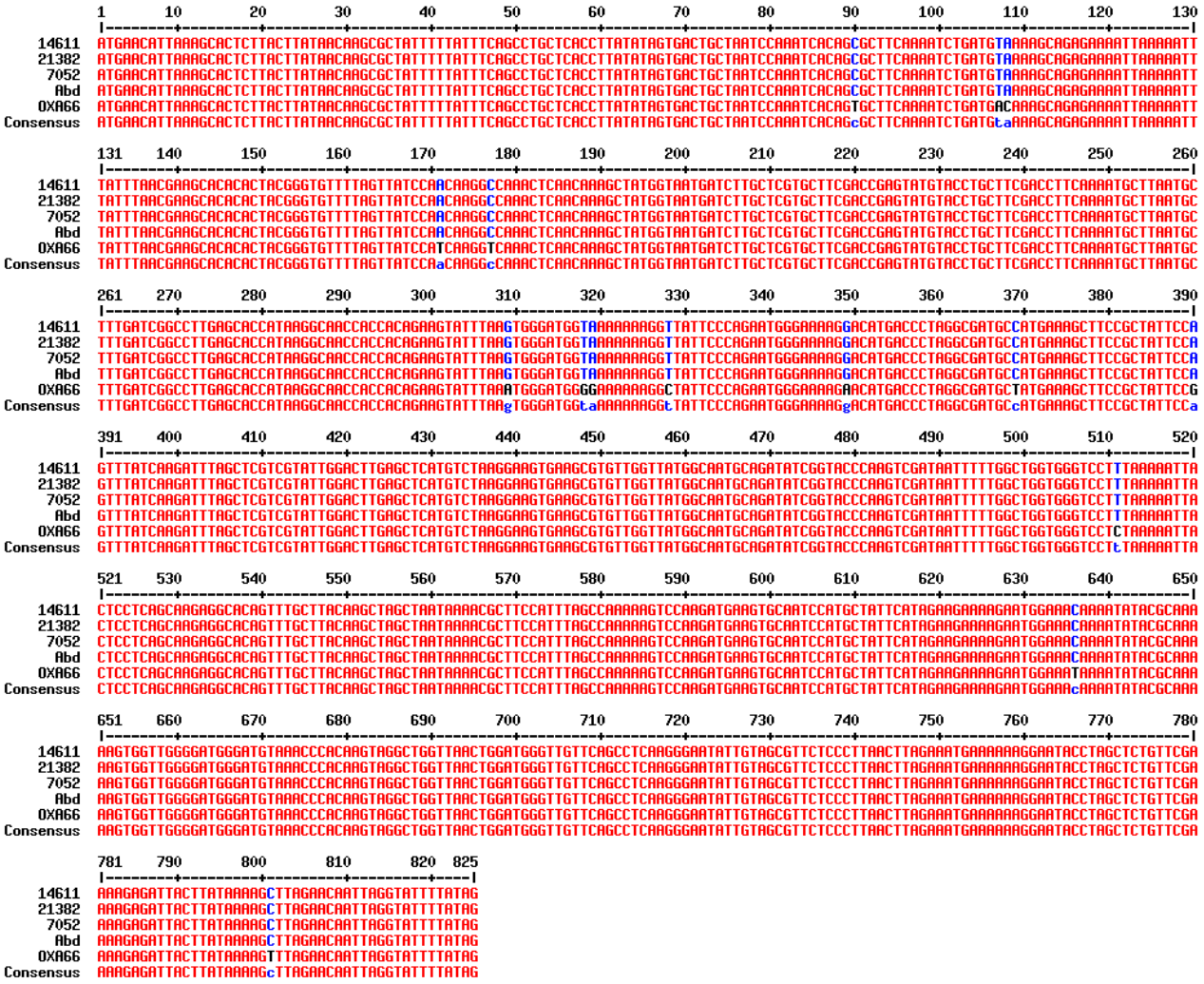


Figure 10: Nucleotide alignment for isolates harbouring *bla*$_{\text{OXA-66}}$

PFGE patterns in figure 11 of the isolates shows that only isolates 21382 and 7052 were clonally related, showing 100% similarity. These isolates were both from patients in the ICU of the same hospital, but in different months: December 2010 and later in May 2011. This similarity may

indicate the persistence of this clone in the ICU, and managed to be transmitted to the patient upon admission.

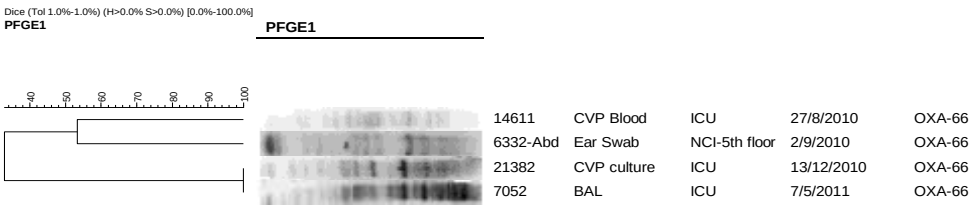


Figure 11: PFGE profiles for *bla*$_{\text{OXA-66}}$ isolates

3.4.4 *bla*$_{\text{OXA-69}}$

Two isolates harbouring *bla*$_{\text{OXA69}}$ were recovered from patients hospitalized in the ICU during the *A. baumannii* outbreak in early 2011.

No silent mutations were found within the sequences (figure 12).

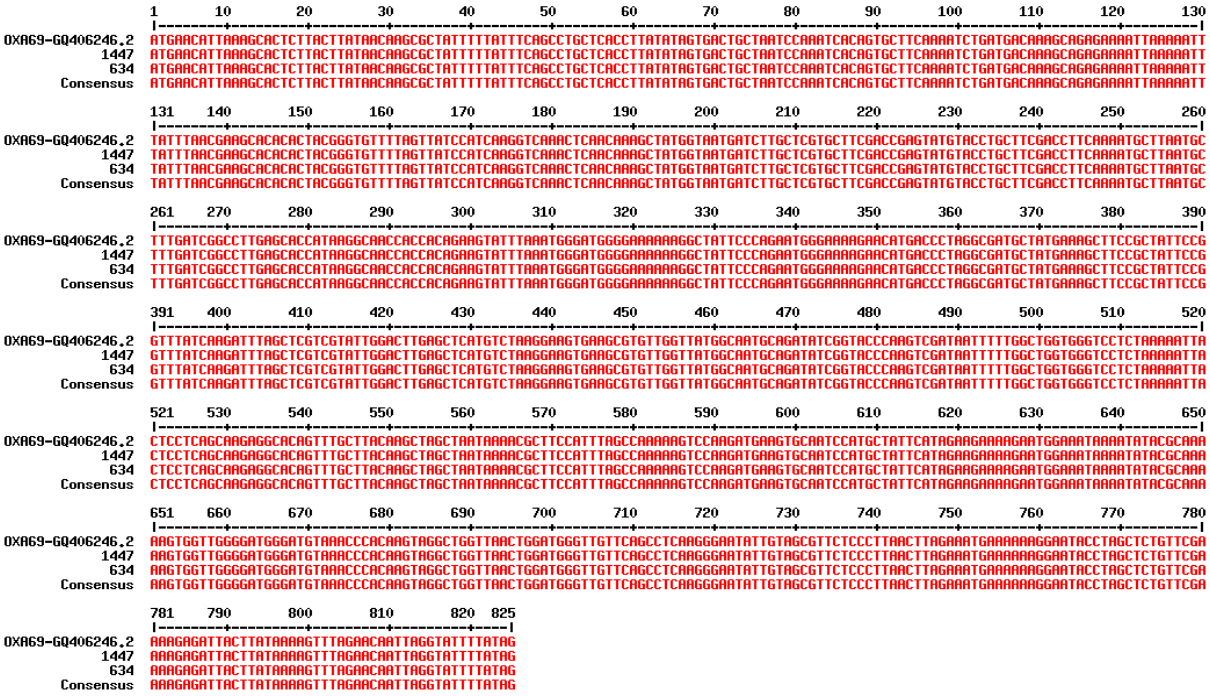


Figure 12: Nucleotide sequence alignment for isolates harbouring *bla*$_{\text{OXA-69}}$

The PFGE profiles (figure 13) of the isolates show a 100% similarity. This correlates with the isolates being in the same ward and isolated in the same outbreak, indicating the presence of a single clone of *bla*$_{\text{OXA-69}}$ in the hospital.

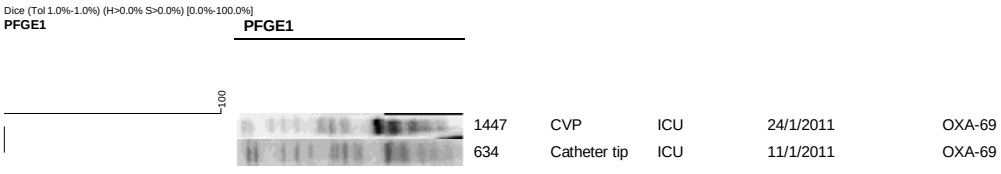


Figure 13: PFGE profiles for *bla*$_{\text{OXA-69}}$ isolates

3.4.5 *bla*$_{\text{OXA-71}}$

Two isolates harbouring *bla*$_{\text{OXA-71}}$ were found in each hospital, CCH and NCI. No silent mutations were found within these sequences (figure 14).

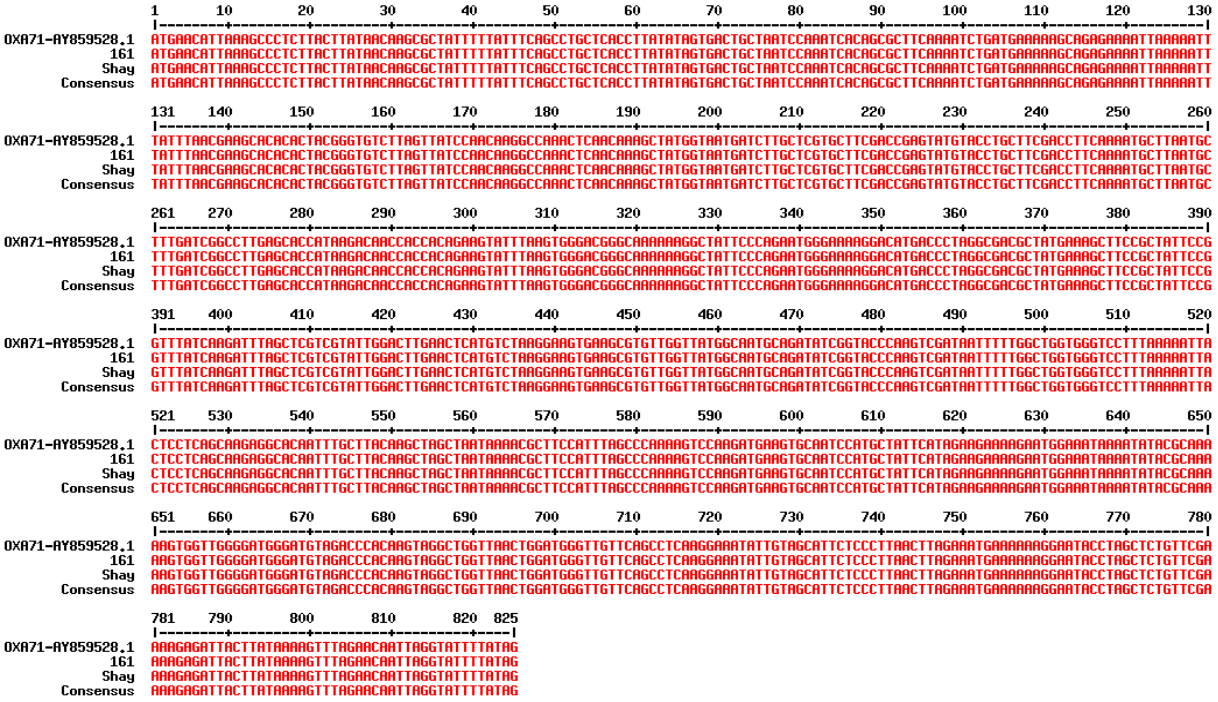


Figure 14: Nucleotide sequence alignment for isolates harbouring *bla*$_{\text{OXA-71}}$

Although the isolates were from different hospitals and different months, their PFGE pattern shows a 100% similarity suggesting clonal relatedness of the strains (figure 15).

Figure 15: PFGE profiles for *bla*$_{\text{OXA-71}}$ isolates

3.4.6 *bla*$_{\text{OXA-78}}$

One isolate harbouring *bla*$_{\text{OXA-78}}$ was recovered from a patient in CCH in December 2010. No silent mutations were identified in the sequence as seen in figure 16.

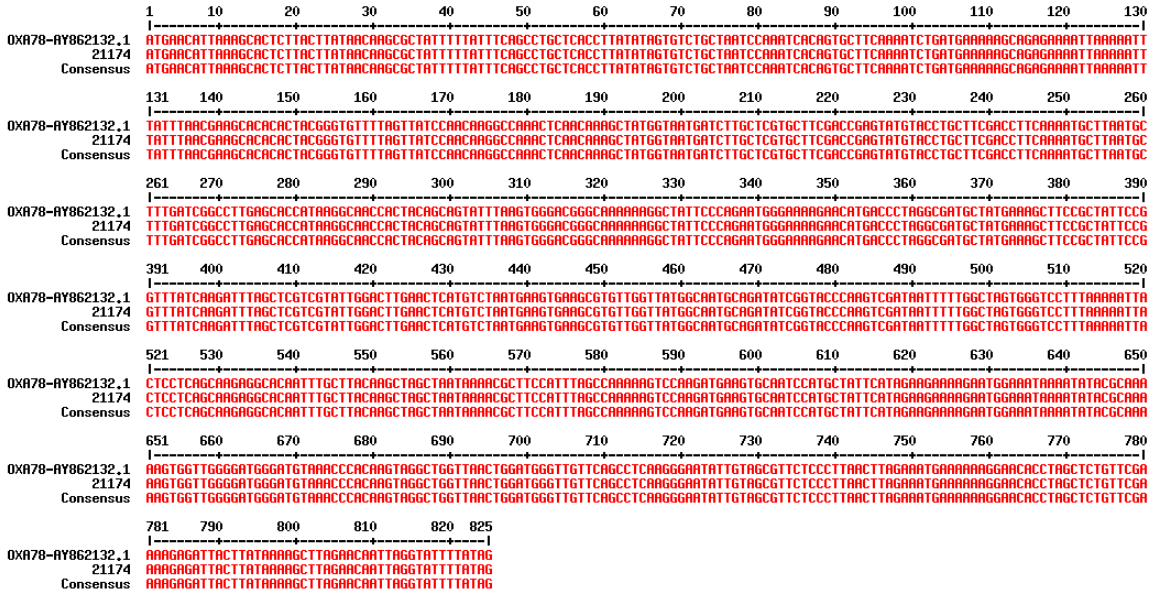


Figure 16: Nucleotide sequence alignment for *bla*$_{\text{OXA-78}}$

3.4.7 *bla*$_{\text{OXA-94}}$

Two isolates from NCI were recovered from the same floor, 1 day apart, were found to have *bla*$_{\text{OXA-94.}}$ No silent mutations were seen in the sequences (figure 17). Surprisingly, their PFGE pattern differs significantly (figure 18), suggesting two different strains.

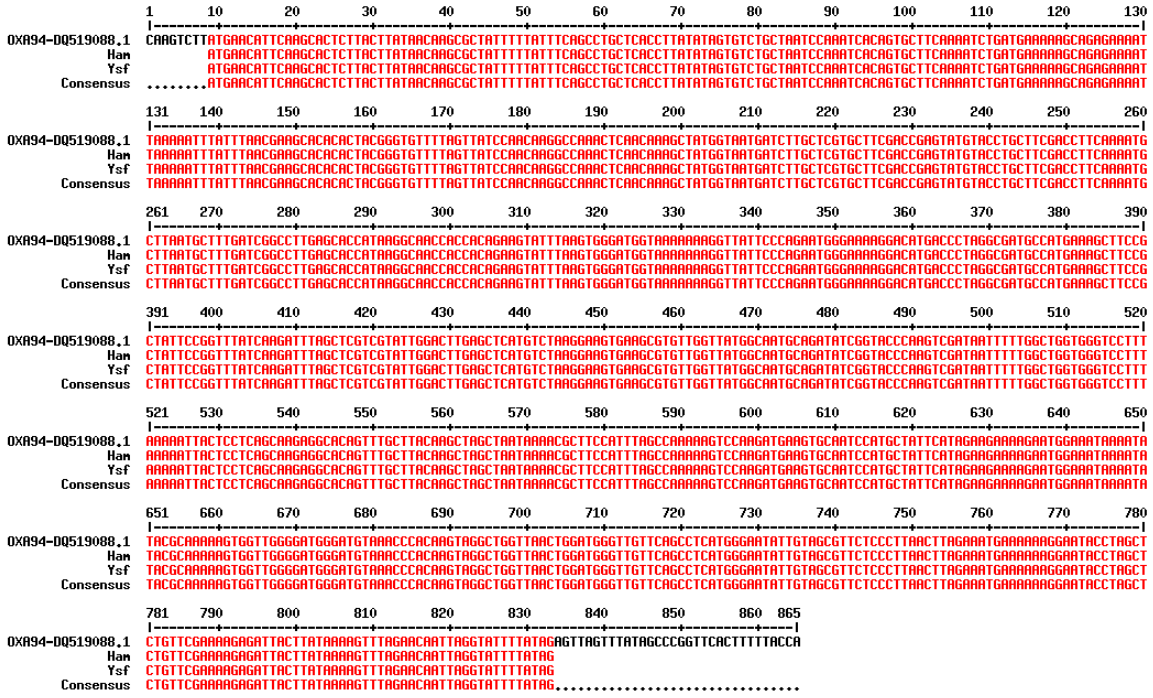


Figure 17: Nucleotide sequence alignment for isolates harbouring *bla*$_{\text{OXA-94}}$

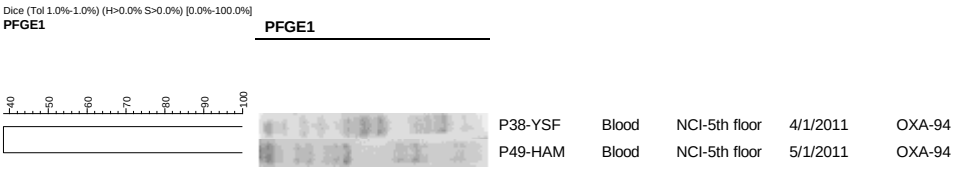


Figure 18: PFGE profiles for *bla*$_{\text{OXA-94}}$ isolates

3.4.8 *bla*$_{\text{OXA-89/100}}$

One isolate, from a patient hospitalized in CCH was found to harbour *bla*$_{\text{OXA89.}}$ No silent mutations were found in the nucleotide sequence (figure19). During amplification, this isolate showed a larger band size for the *bla*$_{\text{OXA-51-like}}$ gene product, suggesting the presence of an

insertion sequence upstream (figure 20). Sequencing revealed the presence of IS*Aba2* directly upstream of the *bla*$_{\text{OXA-89}}$ (figure 21). IS*Aba2* has been associated with the upstream environment and providing suitable promoter sequences to *bla*$_{\text{OXA-58}}$ gene, and this is thus the first identification of IS*Aba2* upstream of *bla*$_{\text{OXA-51-like}}$ gene. The -35 (ttatat) and -10 (ttgtaggat) promoters were 29bp apart, and located102bp and 82bp upstream of *bla*$_{\text{OXA-89}}$ respectively.

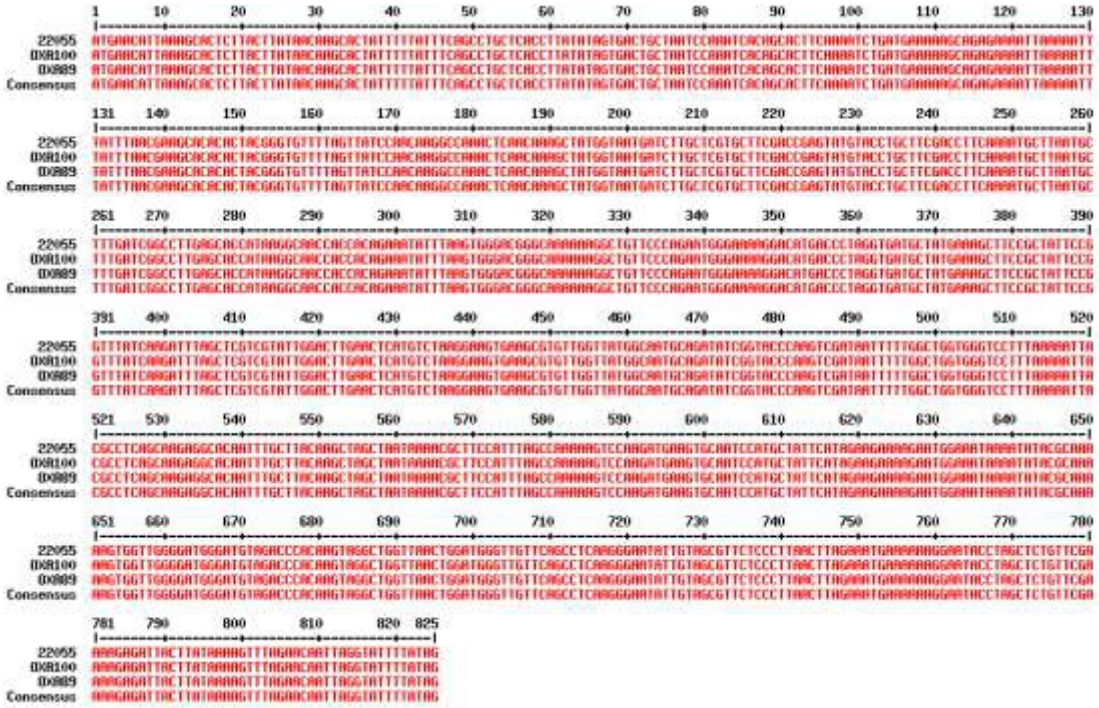


Figure 19: Nucleotide sequence alignment for the isolate harbouring *bla*$_{\text{OXA-89/100}}$

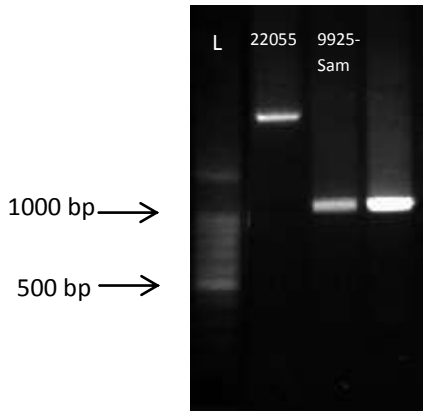


Figure 20: Amplification of *bla*$_{\text{OXA-51-like}}$ genes. Isolate 22055 has a larger band size indicating the presence of an insertion sequence upstream of the gene when compared to isolates 9925-SAM with no IS. L: ladder.

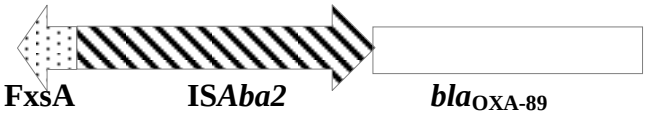


Figure 21: Schematic representation of IS*Aba2* upstream of *bla*$_{\text{OXA-89}}$

3.5 Multi-locus Sequence Typing

A total of 10 isolates, representative of each *bla*$_{\text{OXA-51-like}}$ gene and hospital, were chosen for MLST, to assess whether similar Sequence Types (STs) were present in either hospital, and whether a correlation could be made between a specific ST and *bla*$_{\text{OXA-51-like}}$ genes.

Ten distinct Sequence Types (STs) were identified, seven of which were novel and assigned as ST408-ST414. The remaining three STs were identified as ST331, ST108 and ST208 (Table 21). Typing by MLST further illustrated the large diversity found within the strains, as isolates with similar *bla*$_{\text{OXA-51-like}}$ gene had different STs. Isolates 9925-SAM and P67-AZak, both are from the

NCI and have *bla*$_{OXA-64,}$ but they belong to different STs 409 and 411, respectively. When compared with another *bla*$_{OXA-64}$ positive isolate, 8357, which is from a patient at CCH, we see that another ST is identified in this case: ST408.

ISOLATE NUMBER	*gltA*	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*	ST
8357	18	14	2	28	11	107	5	408
Sam	1	15	2	28	11	58	32	409
1780	24	46	96	11	16	40	26	410
634	10	12	4	11	4	9	5	108
P67 -Azak	13	15	2	28	1	55	32	411
21174	1	34	80	28	1	52	45	412
22055	1	17	6	1	4	116	6	413
161	2	35	45	48	1	11	5	414
P38 - Ysf	28	38	45	1	16	4	2	331
14611	1	3	3	2	2	97	3	208

Table 21: Alleles identified and assigned STs for isolates by MLST.

e-BURST analysis was used to determine evolutionary relationships and clonal complexes within isolates. The analysis uses a default group definition of 6 or more (6/7) shared loci between the STs, with a relaxed definition at 5 or more shared loci out of the 7 (5/7). The analysis was done at the default as well as the relaxed definition and revealed no clonal complexes between the STs and detected 10 singletons.

Despite the diversity, some of the STs identified in this thesis share similarities with each other and other STs from around the world. ST409 and ST411 assigned for isolates 9925-Sam and p67-AZak, respectively, have four similar loci. ST409 shares five alleles with ST35, ST36, ST110 and ST156. ST413 shares six alleles with ST283, whereas it shares five alleles with ST120, ST284 and ST299. ST208 has been previously identified in USA and China, and shares

six alleles with 18 STs including ST74, ST75, ST88, ST89, ST92 and ST118. ST331 has six similar alleles with ST330 and ST332, whereas it shares five alleles with ST65 and ST352. ST108 has six similar alleles with ST109 and ST419. ST108 has five similar alleles to 19 STs including ST109, ST16, ST48, ST95, ST197, ST216 and ST231. ST412 shares six alleles with ST172, and five alleles with ST110, ST169, 224 and 235. ST408, ST410 and ST414 share no similarities with other STs.

The population-snapshot as seen in figure 22 further illustrates the diversity of these isolates as no link can be established between the STs. As seen in figure 22, ST208 is the ancestral ST in its group, from which the related STs are radiating. ST108 is closely related to ST109, which seems to be the ancestral ST in its group. Furthermore, ST419 descended from ST108, according to figure 22. ST331 is closely related to ST330, ST332 and ST65 but the ancestor ST cannot be determined (figure 22). Similar interpretation can be made to ST413 with its relation to ST283, ST284. Other STs identified are widely dispersed in the population snapshot.

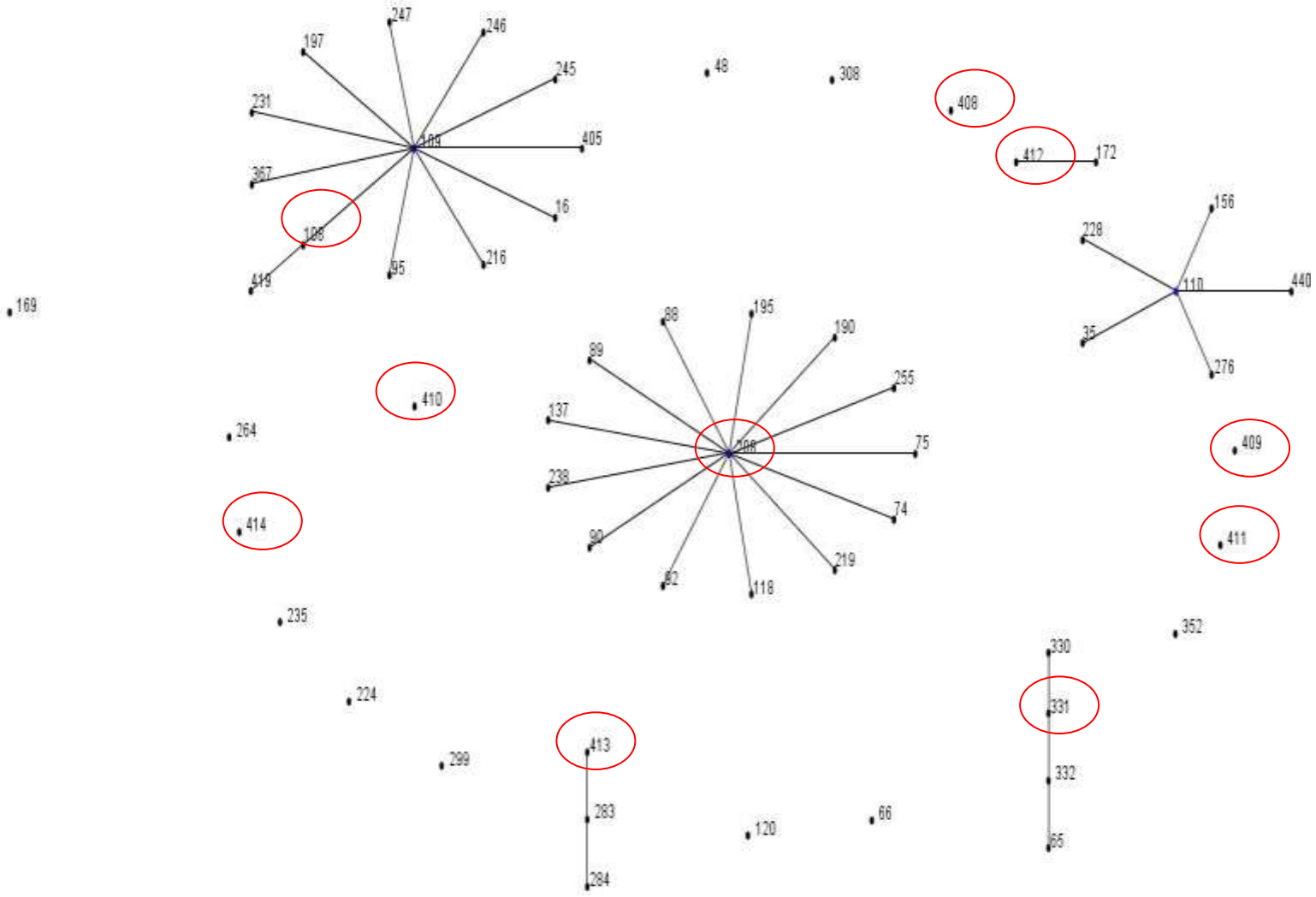


Figure 22: Population snapshot of the e-burst analysis

MLST, on four non-repetitive and distinct clinical strains, isolated in 2004 in the USA, was performed to compare with the diversity of the strains obtained from a single centre in Egypt. The American strains belonged to a single sequence type: ST114 (1, 15, 8, 10, 28, 57, 32). We can hereby see the large diversity of strains in the current collection when compared with other *A. baumannii* strains from another part of the world.

3.5.1 PFGE analysis for MLST

The PFGE pattern of isolates is shown in figure 23. Maximum observed similarity is seen as 60%, thereby further illustrating the epidemiological diversity of the strains.

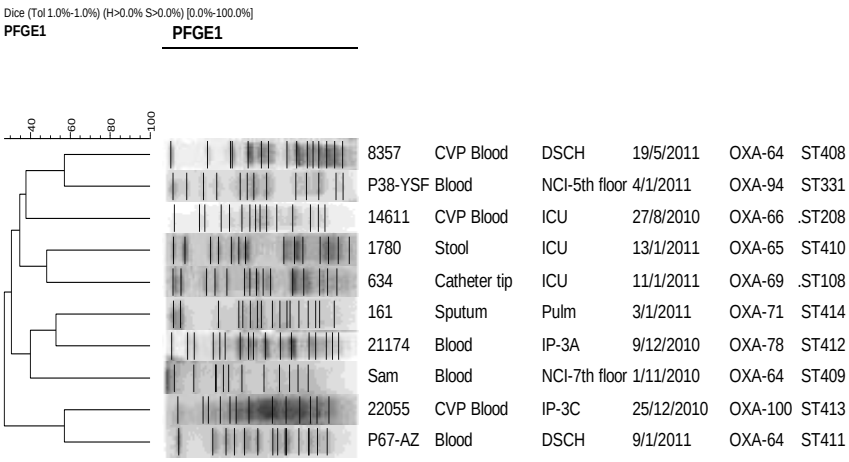


Figure 23: PFGE pattern of isolates typed by MLST, with the site, location of patients, date and OXA-51-like enzymes.

3.6 Class D β-lactamases

Detection of class D β-lactamases was performed by Multiplex PCR according to Woodford *et al.* (2006). Isolates positive for the individual genes were examined further as explained below.

As seen in the figure 24, the *bla*$_{\text{OXA-58}}$ gave an amplicon size of 599 bp, *bla*$_{\text{OXA-23}}$ gene gave an amplicon size of 501 bp, and *bla*$_{\text{OXA-24}}$ gave an amplicon size of 246 bp. The intrinsic *bla*$_{\text{OXA51-like}}$ gene was detected in all *A. baumannii* isolates, with an amplicon size of 353 bp.

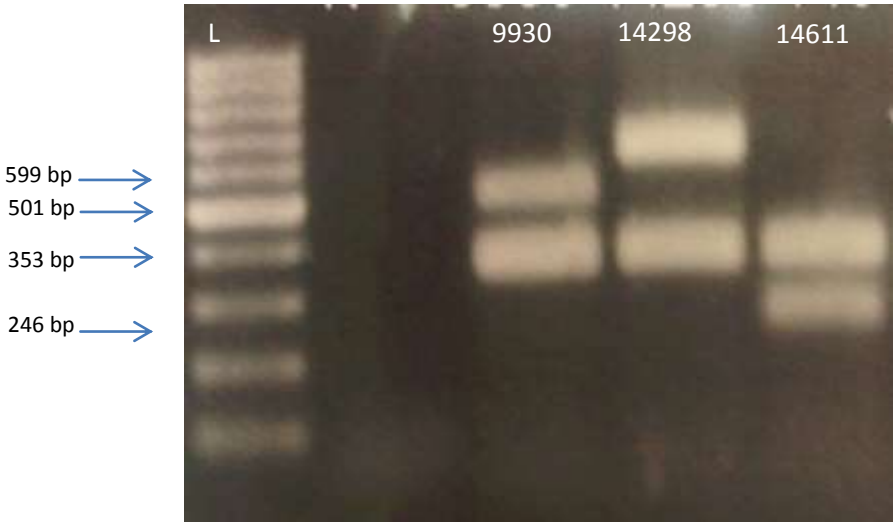


Figure 24: Amplification of Carbapenem-Hydrolysing Class D β-lactamases (CHDL) by PCR and the relative amplicon sizes. L:Ladder, N: Negative Control, Isolate 9930 is positive for *bla*$_{\text{OXA-23}}$, isolate 14298 is positive for *bla*$_{\text{OXA-58}}$ and isolate 14611 is positive for *bla*$_{\text{OXA-40}}$

3.6.1 OXA-23

Eighteen isolates were positive for *bla*$_{\text{OXA-23}}$, they were sequenced in both directions to reveal any insertion sequences upstream as well as to determine the full sequence of the gene.

3.6.1.1 MIC

The MICs of carbapenems for these 18 isolates is listed in the table 22. All isolates harbouring *bla*$_{\text{OXA-23}}$ were resistant with MICs ranging from 8mg/L to 128mg/L, except one isolate: 1447, which was sensitive to carbapenems (MIC <1mg/L). The MIC of meropenem was 4mg/L for some isolates, which is interpreted as intermediate resistance according to the BSAC guidelines. On the other hand, all isolates, except 1447, were resistant to Imipenem.

3.6.1.2 Upstream Environment

Sequencing upstream of *bla*$_{\text{OXA-23}}$ gene revealed the presence of IS*Aba1* in 12 isolates, providing suitable promoter sequences for the transcription of the gene, and thus expressing resistance to carbapenems. High-level resistance was not correlated with the presence of IS*Aba1* in the upstream environment of *bla*$_{\text{OXA-23}}$ in some isolates (table 22) hence suggesting that resistance can be mediated by the presence of the *bla*$_{\text{OXA-23}}$ gene alone. For example, isolate 1780 has a very high MIC to both imipenem and meropenem (MIC = 128mg/L), but does not have IS*Aba1* upstream.

Isolates 1780, 1750, 2106, 1447 and 634 were obtained during an *A. baumannii* outbreak in early 2011. Resistance was mediated by all five isolates harbouring *bla*$_{\text{OXA-23.}}$ Although some isolates were shown to be clonally related, and had similar *bla*$_{\text{OXA-51-like}}$ genes, the upstream environment of *bla*$_{\text{OXA-23}}$ differed significantly. Isolate 1780, 1750 and 2106 were all positive for *bla*$_{\text{OXA-65}}$ gene and shared >80% homology in PFGE, but only 1750 and 2106 had IS*Aba1* upstream of *bla*$_{\text{OXA-23.}}$ MICs were also different for these strains ranging from 8mg/L and 64mg/L in isolates 2106 and 1750 positive for IS*Aba1*, to 128mg/L for isolate 1780 which did not have IS*Aba1* upstream.

ISOLATE NUMBER	HOSPITAL	DATE OF SAMPLE	*bla*$_{\text{OXA-51-like}}$ GENE	IS*Aba1*	MIC - Imipenem mg/L	MIC - Meropenem mg/L
4248	CCHE	15/03/2010	65	+	8	4
4842	CCHE	23/03/2010	65	+	64	32
9930	CCHE	15/06/2010	65		32	16
10262	CCHE	19/06/2010	65	+	64	128
12435	CCHE	23/07/2010	64		16	32
21382	CCHE	13/12/2010	66	+	16	4
634	CCHE	11/01/2011	69	+	8	4
1447	CCHE	24/01/2011	69		0.5	0.06
1780	CCHE	31/01/2011	65		128	128
1750	CCHE	31/01/2011	65	+	32	64
2106	CCHE	08/02/2011	65	+	8	8
2632	CCHE	20/02/2011	65	+	8	8
2625	CCHE	20/02/2011	65		16	16
7052	CCHE	5/7/2011	66	+	8	16
6332-Abd	NCI	02/09/2010	66	+	16	8
461-SF	NCI	15/12/2010	65	+	16	32
9925-Sam	NCI	15/12/2010	64	+	16	8
P391-AH	NCI	14/9/2010	65		8	16

Table 22: *bla*$_{\text{OXA-23}}$ isolates, hospital, date of culture, associated *bla*$_{\text{OXA-51-like}}$ gene, and MIC for carbapenems

3.6.1.3 PFGE for bla$_{OXA-23}$ isolates

There is a large diversity within isolates harbouring *bla*$_{\text{OXA-23,}}$ as seen in the PFGE profile (figure 25) comparison of the isolates below. All isolates from the ICU outbreak in early 2011 are divided into two groups: 1447 and 634 sharing 100% identity in their PFGE pattern, and the other group comprising 2106, 1750 and 1780 sharing <80% similarity (figure 26).

Three clones, although present in different hospitals, have similar PFGE profiles and are positive for *bla*$_{\text{OXA-23.}}$ They were 6332-Abd and 634 and 1447, 461-SF and 4842 CCH, and P391-AH and 10262. That could indicate a clonal dissemination of the strains within hospitals, or transmission by physicians, as some physicians work in both hospitals. Noteworthy, isolate 6332-Abd harboured a different *bla*$_{\text{OXA-51-like}}$ gene when compared to isolates 634 and 1447, however their PFGE patterns are very similar and they were both positive for *bla*$_{\text{OXA-23}}$ gene.

As seen in figure 25, four clonal groups can be seen, sharing >80% homology of their PFGE pattern.

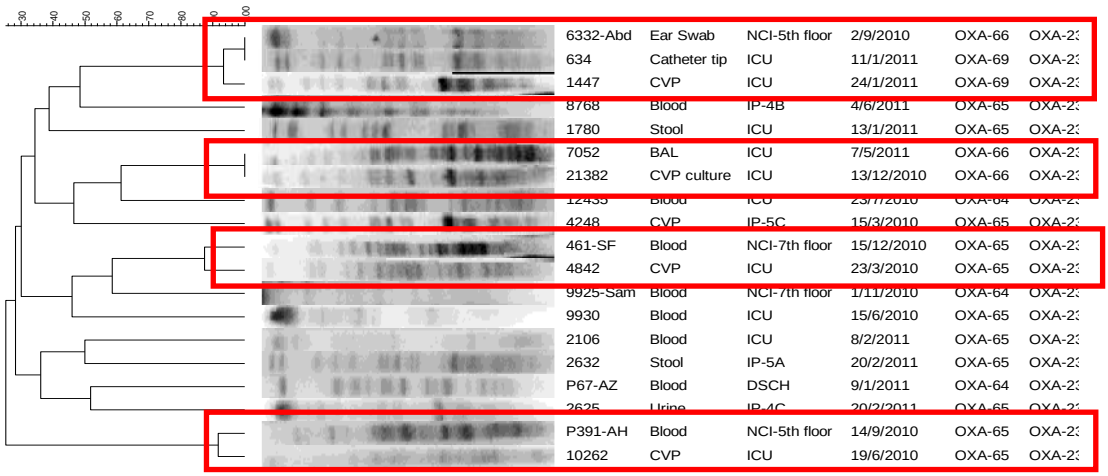


Figure 25: PFGE pattern for *bla*$_{\text{OXA23}}$ isolates

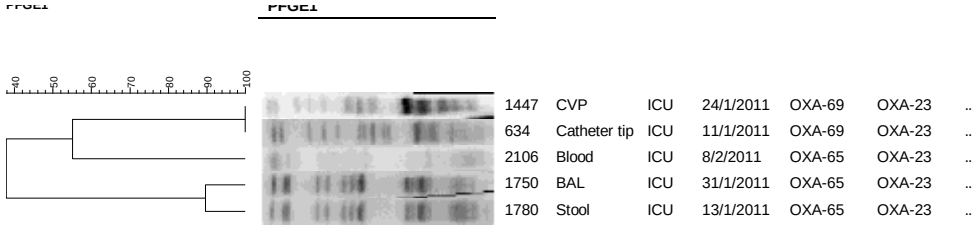


Figure 26: PFGE profiles for isolates from the 2011 ICU outbreak

3.6.2 OXA-24/40

One isolate, 14611, was found positive for *bla*$_{\text{OXA-24/40.}}$ The isolate was from a CVP culture of a 15 month old patient in the ICU at CCH.

3.6.2.1 Upstream Environment and localization

IS*Aba1*, IS*Aba2* and IS*Aba3* were screened for, but no insertion sequence was detected upstream of the *bla*$_{\text{OXA-24/40}}$ gene.

S1 nuclease digestion detected no plasmids in this isolate, hereby suggesting the chromosomal location of the *bla*$_{\text{OXA-24/40}}$ gene.

3.6.2.2 MIC

The isolate was highly resistant to carbapenems, with MICs of 128mg/L and 32 mg/L for Imipenem and Meropenem, respectively.

3.6.3 OXA-58

Five isolates were positive for *bla*$_{\text{OXA-58.}}$ They were isolates from both hospitals, in several wards and different times of the year. From CCH, isolate 14298 was from a catheter tip culture of a Hodgkin's disease patient obtained in August 2010 whereas isolate 2625 was from an Ewing

Sarcoma patient in February 2011. As for isolates from the NCI, P67-AZak and P49-Ham were both blood cultures isolated in January of 2011, but in different wards. 679-Bas was an ear swab culture in September 2011.

3.6.3.1 MIC

As shown in table 23, all isolates were resistant to Imipenem and Meropenem with MICs ranging from 8mg/L to 64mg/L, except isolate 14298 which was sensitive to Meropenem (MIC 2mg/L).

ISOLATE NUMBER	Hospital	DATE OF SAMPLE	*bla* OXA-51-like GENE	Upstream *bla* OXA-58	MIC - Imipenem	MIC - Meropenem
14298	CCHE	22/08/2010	64	ISAba3-IS1006	8	2
2625	CCHE	20/02/2011	65		16	16
Bas	NCI	04/09/2010	64		8	8
P49 - Ham	NCI	05/01/2011	94		8	8
P67 - Azak	NCI	09/01/2011	64	ISAba3-IS1008	64	16

Table 23: *bla*$_{OXA58}$ isolates: associated *bla*$_{OXA51\text{-}like}$ gene, insertions upstream and MIC for carbapenems

3.6.3.2 Upstream environment of bla$_{OXA-58}$

The *bla*$_{OXA-58}$ gene was bracketed by IS*Aba3* in all isolates. Larger product sizes were observed for two isolates (figure 27), and sequencing upstream of *bla*$_{OXA-58}$ revealed the presence of IS*Aba3* interrupted by two insertion sequences: IS*1006* in one isolate: 14298 (from CCH), and IS*1008* in P67-AZak (from the NCI). Using BProm (Softberry, Inc., Mount Kisco, NY) promoter sequences for 14298 were located in IS*1006* with -35 (TTGCA) and -10 (TGGTAAGCT) 17bp apart and located 807bp and 786bp upstream of *bla*$_{OXA-58.}$ As for isolate P67-AZak, promoter

sequences were also located in IS*1008* with –35 (ATCACA) and -10 (TTTTATACT) 20bp apart and located 548bp and 568bp upstream of $bla_{OXA58.}$ The current results show that the promoter sequences are provided solely by the IS*1006* and IS*1008* sequences, rather than forming a hybrid promoter with IS*Aba3*. RT-PCR results showed no difference in gene expression level between the isolates, with a ratio of ~1, even when compared to an isolate harbouring IS*Aba3* alone in the upstream region.

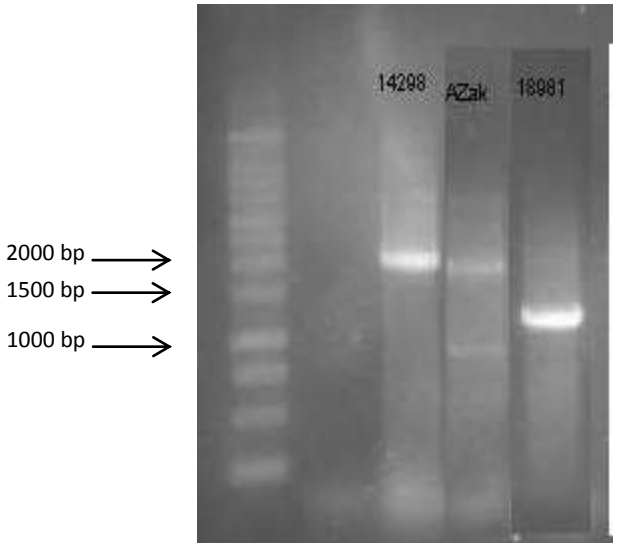


Figure 27: Amplification of upstream region for bla_{OXA-58}. Strain 14298 &AZak harbour an interrupted IS*Aba3*, giving a larger amplicon than strain 18981 (not in strain collection), harbouring IS*Aba3* only.

IS*1008* and IS*1006* both belong to IS*6* family. They do however differ in 15 amino acids, as shown in figure 28. The nucleotide alignment significantly differs between the sequences as well (figure 29). The location of promoters hence differs in the sequences and no alignment can be inferred. Although part of the same family, IS*1006* and IS*1008* appear to act with different mechanisms and have distinct functions, mainly in their contribution in resistance to carbapenems. IS*1008* seems to provide a stronger promoter for the expression of the bla_{OXA-58}

gene, as the MIC to imipenem and meropenem is 64mg/L and 16mg/L, respectively. Whereas in isolate 14298, the imipenem MIC was significantly less: 8mg/L and it was intermediate to meropenem. The sequences, however, appear to transpose in a similar manner and interrupt the IS*Aba3* sequence approximately after 350bp. Additionally, neither IS*1006* nor IS*1008* act as hybrid promoters and interrupt the promoter sequences of IS*Aba3* upon transposition.

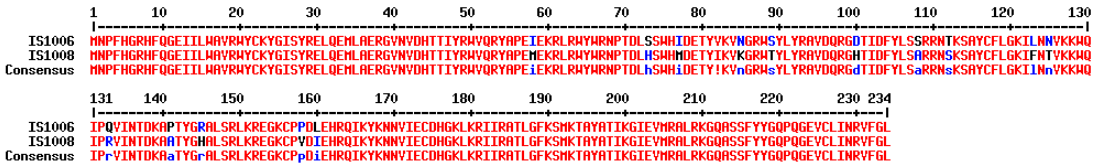


Figure 28: Protein Sequence alignment of IS*1006* and IS*1008*

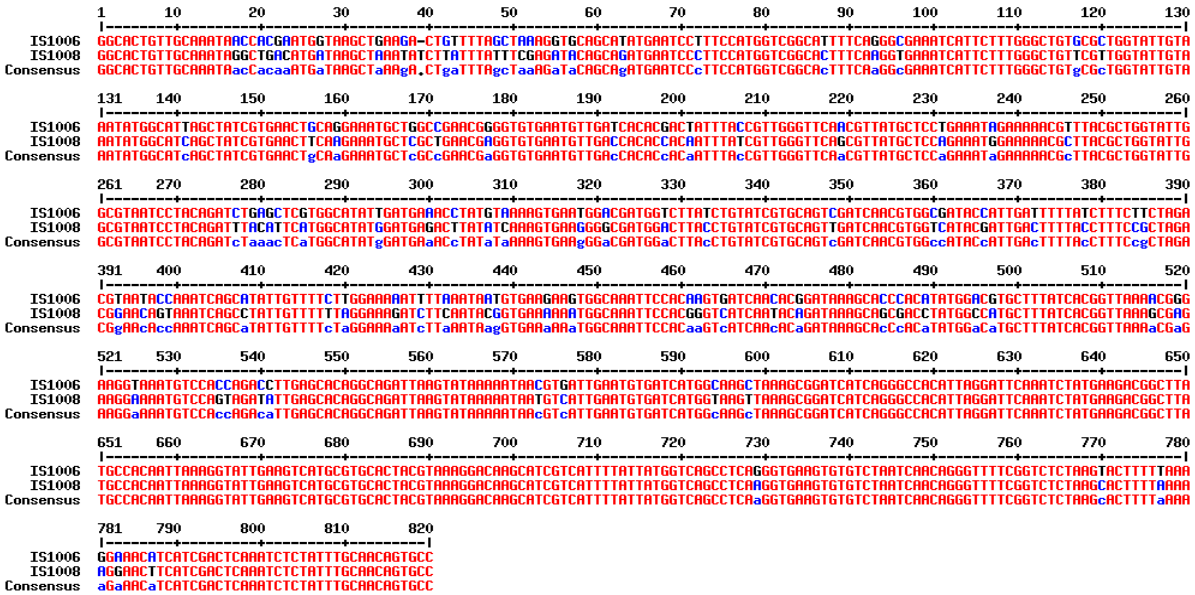


Figure 29: Nucleotide Sequence Alignment of IS*1006* and IS*1008*.

3.6.3.3 Plasmids and localization of bla$_{OXA-58}$

S1 nuclease digestion of isolates 14298 and P67-AZak revealed the presence of 3 plasmids at 50 kb, 60 kb and 100 kb. Hybridization of *bla*$_{\text{OXA-58}}$ to these plasmids revealed that the gene was located on the 50 kb plasmid. This may indicate that a similar plasmid with *bla*$_{\text{OXA-58}}$ is circulating in the isolates.

3.6.3.4 PFGE analysis for bla$_{OXA-58}$

PFGE analysis of *bla*$_{\text{OXA58}}$ isolates showed a large diversity among the strains, with the maximum similarity at 60% (figure 30). Noticeably, isolates 14298 and P67-AZ have similar patterns, although they were from different hospitals and harboured a distinct upstream environment. This further confirms the localization of *bla*$_{\text{OXA-58}}$ on plasmids, hence facilitating the dissemination of the gene into different clones and hospitals.

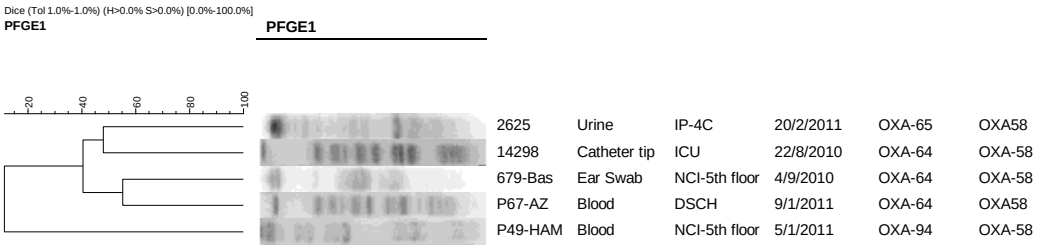


Figure 30: PFGE pattern for *bla*$_{\text{OXA-58}}$ isolates

3.7 Extended-Spectrum β-lactamases (ESBLs)

Screening for the presence of an ESBL was performed for isolates resistant to ceftazidime. All isolates were negative for SHV, CTX-M and VEB. Positive results are described below.

3.7.1 PER-like enzymes

Three different *bla*$_{\text{PER-like}}$ genes: *bla*$_{\text{PER-1}}$, *bla*$_{\text{PER-3}}$ and *bla*$_{\text{PER-7}}$ were identified in 3 different isolates, from both hospitals.

Figure 31 shows the amino acid sequence comparison of the three PER-like enzymes identified. PER-3 is a point-mutant derivative of PER-1, whereas PER-7 contains two amino acid substitutions.

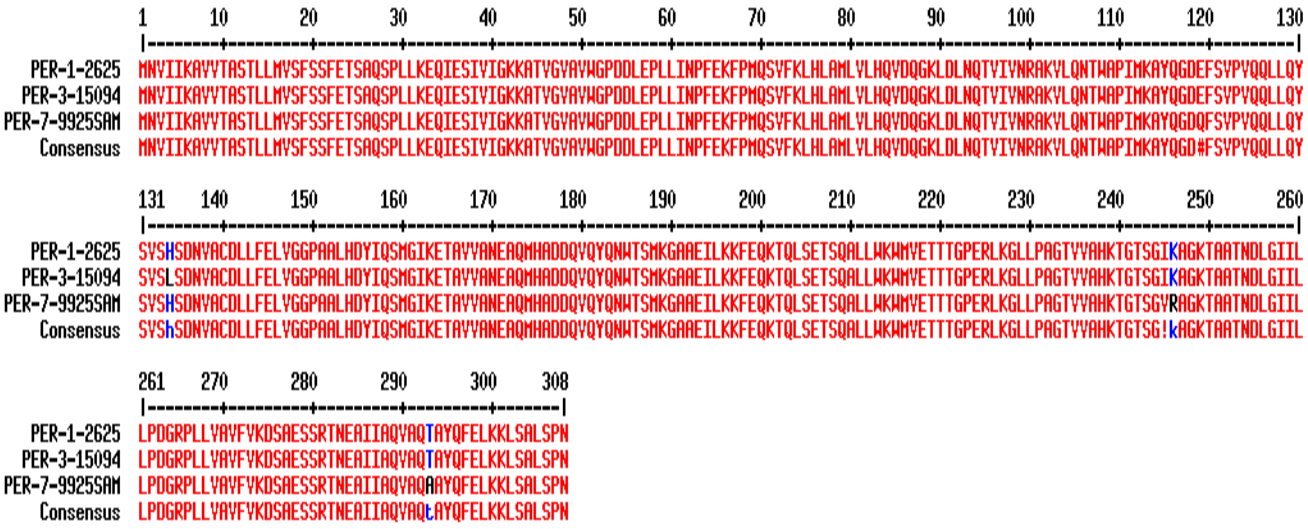


Figure 31: Protein alignment of PER-like enzymes identified

3.7.1.1 PER-1

bla$_{PER-1}$ was identified in isolate 2625 from CCH. It was from a urine sample of a 15-year old Ewing Sarcoma patient. The same isolate was positive for *bla*$_{OXA-58}$ and harboured the intrinsic *bla*$_{OXA-65}$ gene.

The MIC of ceftazidime was >256mg/L. Phenotypic tests confirmed the presence of an ESBL. PCR was positive for *bla*$_{PER-like}$ genes and sequencing revealed the presence of *bla*$_{PER-1}$ gene.

3.7.1.1.1 The genetic environment of bla$_{PER-1}$

The genetic environment of *bla*$_{PER-1}$ gene was analysed and showed that the gene was flanked by two insertion sequences: IS*Pa12* upstream and IS*Pa13* downstream (figure 32). This is similar to results published by Poirel *et al*. (2005), who in their report analysed the genetic environment of *bla*$_{PER-1}$ from different Gram-negative organisms and found the gene to be part of a composite transposon, flanked by these two insertion sequences.

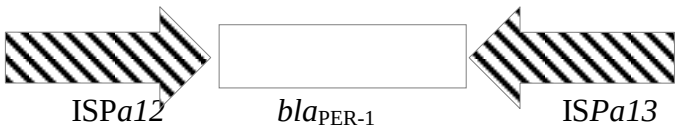


Figure 32: Schematic representation of the genetic environment of *bla*$_{PER-1}$

IS*Pa12* and IS*Pa13* both belong to the same IS*4*family. They do however differ significantly in their amino acid with 63% similarity (figure 33).

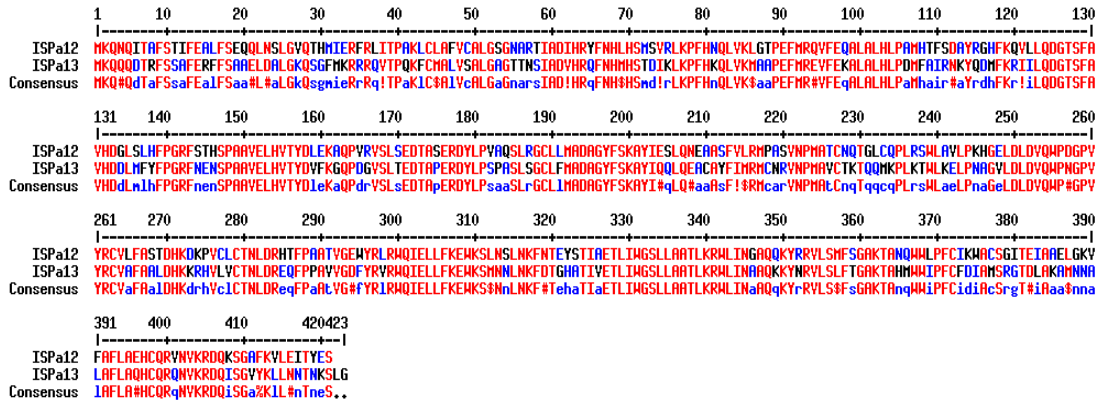


Figure 33: Protein sequence alignment for IS*Pa12* and IS*Pa13*

3.7.1.2 PER-3

bla$_{\text{PER-3}}$ has only been found in *Aeromonas punctata* (Wu *et al.* 2011). This is thus the first identification of this gene in *A. baumannii*. It was found in a urine culture of a 9-year old Osteosarcoma patient in CCH.

Isolates 15094 was resistant to ceftazidime with MIC of 64 mg/L. The intrinsic *bla*$_{\text{OXA-51-like}}$ gene was identified as *bla*$_{\text{OXA-65.}}$ The isolate was however sensitive to carbapenems, and did not harbour any of the acquired class-D carbapenemases.

3.7.1.2.1 Upstream environment of bla$_{PER-3}$

The upstream environment of *bla*$_{\text{PER-3}}$ was sequenced and revealed the following structure: A complex-integron-class-1 structure, harbouring *sul*1, *qacEΔ-1* in the variable 3'CS and orf*513* directly upstream of the *bla*$_{\text{PER-3}}$ gene, serving as the transcriptional promoter (figure 34).

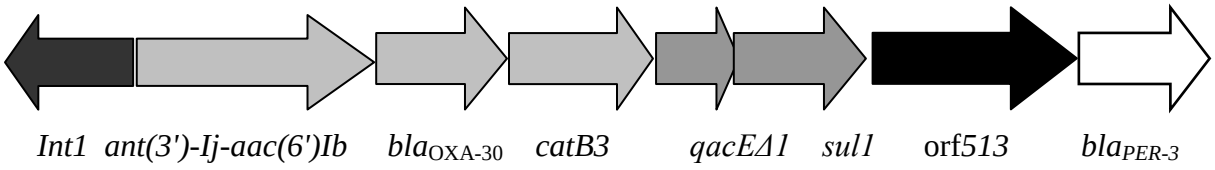


Figure 34: Schematic representation of the genetic environment of *bla*$_{\text{PER-3}}$

3.7.1.2.2 Localization of bla$_{PER-3}$

Previous reports have shown that *bla*$_{\text{PER-3}}$ gene could be localized on both plasmids and chromosomes (Wu *et al*., 2011) therefore identification of the location of the gene was attempted with this isolate.

The S1 nuclease digestion revealed the presence of three plasmids, the sizes of which were 60, 70 and 145.5 kb, respectively. There was no hybridization of *bla*$_{\text{PER-3}}$ specific probes to any of these plasmids, suggesting chromosomal localization of the gene.

3.7.1.2.3 Plasmid curing and conjugation

Plasmid curing was done to confirm the chromosomal localization of the gene. Ceftazidime susceptibility was tested after 10 days of curing, and the zone of inhibition did not increase showing no loss of resistance, thus endorsing the chromosomal localization. Furthermore, attempted conjugal transfer was also negative.

3.7.1.3 PER-7

bla$_{PER-7}$ has been reported in France (Bonnin *et al.,* 2011) and the UAE (Opazo *et al.,* 2012a). Isolate 9925-SAM from the NCI was found to be positive to this gene.

The MIC of Ceftazidime was 256 mg/L and the isolate had the intrinsic *bla*$_{OXA-64}$ gene. MLST identified a novel ST for this isolate, assigned ST409. The isolate was furthermore positive for *bla*$_{OXA-23,}$ and was also resistant to carbapenems.

3.7.1 3.1 Upstream environment of bla$_{PER-7}$

The upstream environment of *bla*$_{PER-7}$ was identical to that reported by Opazo *et al*. (2012a) in that it was located in a complex class-1 integron, with *qacEΔ1*, *sul1* in the variable region, and orf*513* serving as the promoter of the gene. Downstream of *bla*$_{PER-7}$ was a *gst* gene (figure 35).

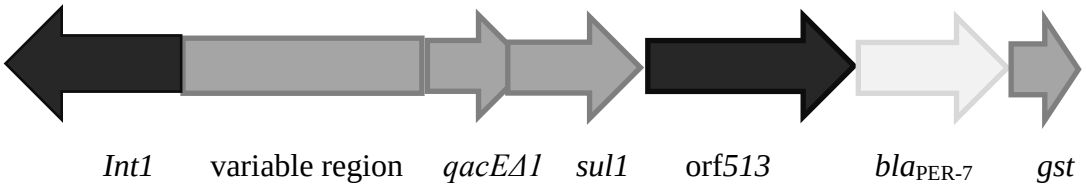


Figure 35: Schematic Representation of the genetic environment of *bla*$_{PER-7}$

3.7.1.3.2 Localization of bla$_{PER-7}$

S1 nuclease digestion revealed 4 plasmids for the isolate. The sizes of these plasmids were approximately 60 kb, 140 kb, 145.5 kb and ~155 kb.

Upon plasmid extraction from the gel, hybridization of PER-7 was attempted and it was positive for the ~155 kb plasmid.

Plasmid curing was also performed on isolate 9925-Sam to confirm the plasmid localization of the *bla*$_{\text{PER-7}}$ gene, and after 10 days of curing, the ceftazidime zone of inhibition increased from 1mm to 5mm, thereby confirming the localization on the lost plasmid.

3.7.2 TEM-like enzymes

TEM-like enzymes are widely found in *A. baumannii* around the world and confer resistance to Ceftazdime as well as other third and fourth generation cephalosporins. .

Six isolates were positive for *bla*$_{\text{TEM-like}}$ PCR and sequencing of the gene revealed that they all harboured the *bla*$_{\text{TEM-1}}$ gene, a narrow spectrum β-lactamase. TEM-1 is a penicillinase, and is not capable of hydrolysing third-generation cephalosporins (Woodford & Ellington, 2007). Only TEM-like variants have evolved to hydrolyse third-generation cephalosporins. A disk diffusion assay to test susceptibility to ampicillin was performed and revealed that all six isolates had no zone of inhibition.

3.7.3 *bla*$_{\text{ADC}}$

ADCs are naturally occurring cephalosporinases in *A. baumannii*, the overproduction of which causes resistance to cephalosporins.

All isolates harboured ADCs, and 6 isolates had IS*Aba1* upstream of the *bla*$_{ADC}$ gene. The presence of the insertion sequence upstream correlated with high resistance to ceftazidime as seen in table 24.

ISOLATE	MIC - CAZ
7052	>256mg/L
634	>256mg/L
21382	>256mg/L
1447	>256 mg/L
Abd	>256mg/L
14611	>256mg/L

Table 24: Isolates harbouring IS*Aba1* upstream of *bla*$_{ADC}$ and the associated ceftazidime MIC

No other mechanism of ceftazidime of resistance was detected in the isolates harbouring IS*Aba1* upstream of the *bla*$_{ADC}$ gene, suggesting the overexpression of *bla*$_{ADC}$ is the sole resistance determinant.

Furthermore, the overexpression of *bla*$_{ADC}$ seems to correlate with *bla*$_{OXA-23}$, where five of the isolates harbour the acquired *bla*$_{OXA-23}$ gene contributing to carbapenem resistance. Only isolate 14611 harboured *bla*$_{OXA-24/40.}$ Interestingly, the overexpression of *bla*$_{ADC}$ was associated with isolates harbouring *bla*$_{OXA-66}$ and *bla*$_{OXA-69}$ only.

3.8 PFGE profile analysis

Banding pattern similarity was assessed using the Dice co-efficient. Overall similarity was calculated as 28.69%. A large variability is seen in the PFGE profiles of the isolates, with only 6 groups of 2 isolates sharing >80% similarity (figure 36). The majority of isolates had <60% similarity, even if they harboured the same *bla*$_{\text{OXA51-like}}$ gene. The results indicate the presence of several clones from different origins in the hospital. This is particularly apparent given the clonal diversity found within one ward at the hospital.

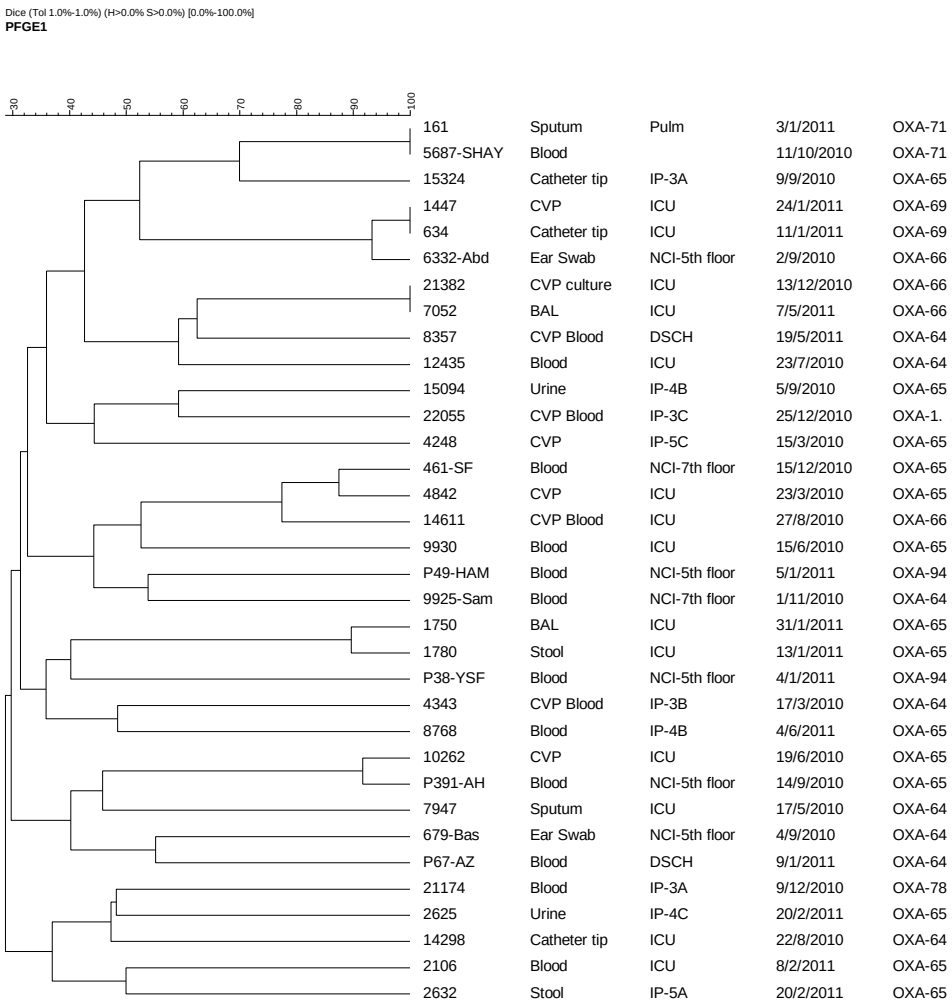


Figure 36: PFGE pattern for all isolates, location of patients, date of isolate, *bla*$_{\text{OXA-51-like}}$ gene, CHDL and *bla*$_{\text{ADC}}$

3.8.1 Diversity in the NCI

The isolates obtained from patients at the NCI were localized in that most of them were recovered from patients on one floor of the hospital, with the exception of two isolates. The

PFGE profiles of these isolates were very diverse, and also showed the presence of several clones in this case not only within the hospital, but in one floor of that hospital (figure 37).

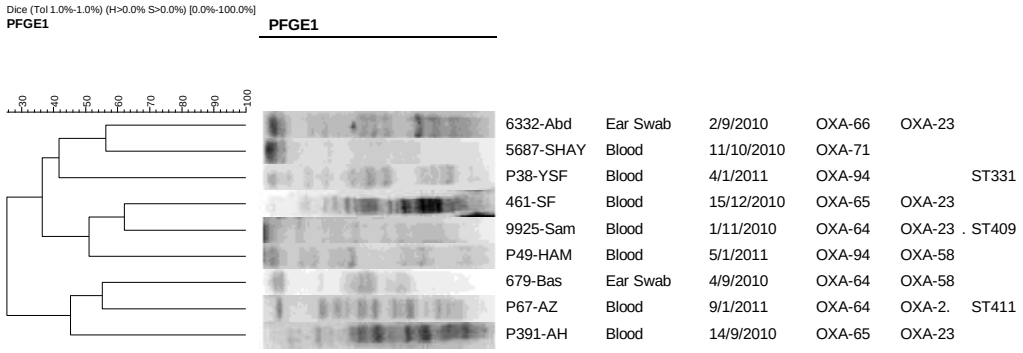


Figure 37: PFGE pattern for isolates from the NCI, site of isolate, location of patients, date of isolate, *bla*$_{\text{OXA-51-like}}$ gene, CHDL and ST.

3.8.2 Association of *bla*$_{\text{OXA-51-like}}$ clones at CCH with governorates

Given the large diversity within strains from CCH as well as the different governorates from which patients originate, a comparison using the PFGE profile and *bla*$_{\text{OXA-51-like}}$ gene was carried out to assess whether a particular clone originated from a particular governorate and subsequently brought to the hospital by the patient.

3.8.2.1 Alexandria

Two patients from Alexandria had *A. baumannii* infections at CCH. They had different $bla_{\text{OXA-51-like}}$ genes and were epidemiologically different according to their PFGE patterns as seen in figure 38. It can therefore not be concluded that the strains were brought by the patients.

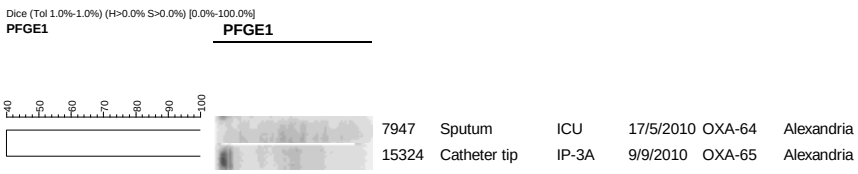


Figure 38: PFGE pattern of isolates obtained from patients form Alexandria

3.8.2.2 Bani Sweif

Two patients from Bani Sweif were found to harbour *A. baumannii* with $bla_{\text{OXA-65}}$ as their intrinsic genes. Although the isolates were recovered on closely related dates (Figure 39) the patients were hospitalized in different wards at the hospital. Despite having similar $bla_{\text{OXA-51-like}}$ genes, their PFGE profiles indicate the isolates are epidemiologically distinct.

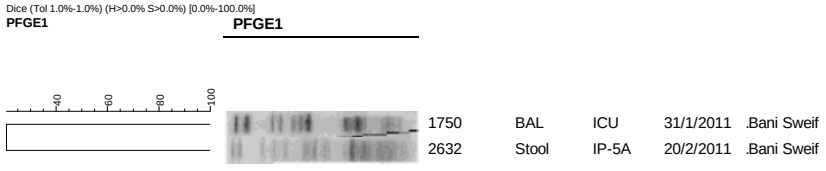


Figure 39: PFGE pattern of isolates from patients from Bani Sweif

3.8.2.3 Cairo

Similar to the results seen above, isolates obtained from patients originating from Cairo have similar *bla*$_{\text{OXA-51-like}}$ genes: *bla*$_{\text{OXA-65,}}$ but have distinct PFGE profiles (figure 40).

Figure 40: PFGE patterns of isolates obtained from patients from Cairo

3.8.2.4 El Dakahleya

Four isolates obtained from patients originating from El-Dakahleya identified 2 intrinsic genes: *bla*$_{\text{OXA-66}}$ and *bla*$_{\text{OXA65.}}$ Two isolates: 21382 and 7052 were both located in the ICU but in different months, they both harboured *bla*$_{\text{OXA-66}}$ and had identical PFGE profiles (figure 41). This may indicate they were brought from the patients into the hospital, which is hence representative of the clones present in El Dakahleya. On the other hand, although isolate 14611 was also positive for *bla*$_{\text{OXA-66}}$, it had a very distinct PFGE profile. Some similarity to isolate 4248, positive for *bla*$_{\text{OXA-65}}$ was seen when PFGE profile was compared with that of 21382 and 7052, which may also indicate a common origin from which clones evolved in that governorate.

Figure 41: PFGE patterns of isolates obtained from patients from El Dakahleya

3.8.2.5 El Fayoum

Two patients from El Fayoum were both hospitalized in the ICU but in different months. They both had *bla*$_{\text{OXA-65}}$ as the intrinsic enzyme but very distinct PFGE profiles (figure 42). No epidemiological link can be established between these strains, as they seem to originate from different origins.

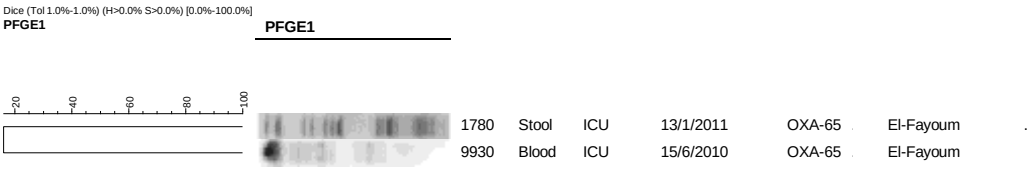


Figure 42: PFGE patterns of isolates obtained from patients from El Fayoum

3.8.2.6 El Sharkeya

Two patients from El-Sharkeya were infected with two distinct *A. baumannii* isolates harbouring $bla_{\text{OXA-78}}$ and $bla_{\text{OXA-64.}}$ The difference in PFGE pattern (figure 43), $bla_{\text{OXA-51-like}}$, location and date of infection indicated that the isolates did not have a common origin, and they cannot be attributed to the governorate from which the patients were from.

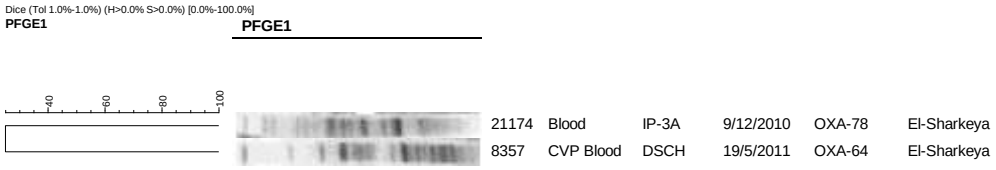


Figure 43: PFGE patterns of isolates obtained from patients from El Sharkeya

3.8.2.7 Giza

Four isolates were recovered from patients from Giza, with 3 genes identified: $bla_{\text{OXA-65}}$, $bla_{\text{OXA-89/100}}$ and $bla_{\text{OXA-69}}$. Isolates 1447 and 634 were part of the ICU outbreak and given their identical PFGE pattern, they seem to have been clonally related and of similar origin. Strain 634, which was isolated first, was probably transferred in the ICU by a healthcare worker and hence caused the infection in another patient, yielding strain 1447 two weeks later. Additionally, some homology in PFGE profile is seen to strain 15094, which may again postulate the possibility a common evolutionary origin (figure 44).

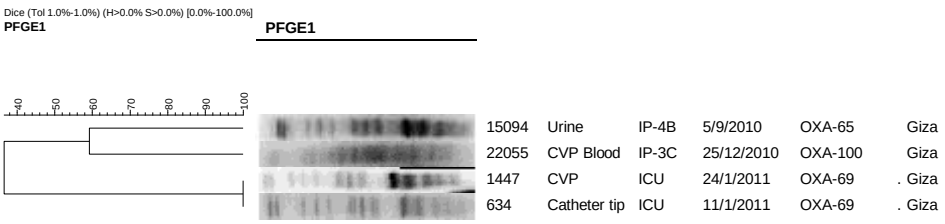


Figure 44: PFGE patterns of isolates obtained from patients from Giza

3.8.2.8 Qina

Two genetically and epidemiologically distinct isolates were recovered from patients from Qina. *bla*$_{\text{OXA-64}}$ and *bla*$_{\text{OXA-71}}$ genes have been reported to be closely related enzymes (Evans *et al*. 2008) which therefore might mean that both isolates have evolved separately but from a common origin. Figure 45 shows the distinct patterns of the isolates.

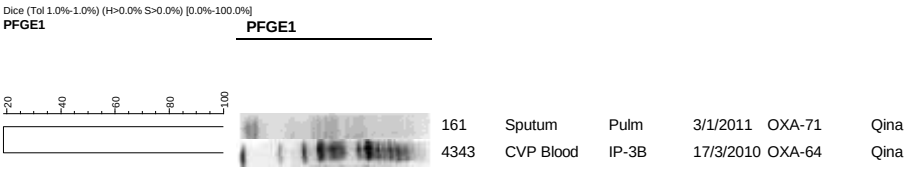


Figure 45: PFGE patterns of isolates obtained from patients from Qina

3.9 Carbapenem susceptibility

A total of 16 and 18 isolates were resistant to imipenem and meropenem by disk-diffusion assay, respectively.

A total of 26 isolates representing 76% were resistant to either Imipenem, Meropenem or both (MIC ≥8mg/L) according to the BSAC guidelines. Four isolates (4248, 634, 21382 and 14298) were resistant to Imipenem but intermediate to Meropenem. Eight isolates were sensitive to both Imipenem and Meropenem.

Resistance to either Imipenem or Meropenem was associated with the presence of CHDL as stated previously. Isolates 4248, 634, 21283, which were resistant to Imipenem and intermediate to Meropenem, encoded *bla*$_{\text{OXA-23}}$ with an associated IS*Aba1* upstream, whereas isolate 14298 encoded *bla*$_{\text{OXA-58}}$ with a hybrid promoter IS*Aba3*-IS*1008* upstream. This means that the presence of insertion elements upstream of resistance genes did not result in resistance to all classes of carbapenems. Additionally, for isolate 22055, carbapenem resistance was attributed to the presence of IS*Aba2* upstream of *bla*$_{\text{OXA-89/100,}}$ as no CHDL was detected.

Sensitive isolates contained no CHDL, with the exception for isolate 1447, which was positive for *bla*$_{\text{OXA-23}}$ but the MICs for of Imipenem and Meropenem were 0.5 and 0.06 mg/L, respectively.

3.10 Metallo β-lactamases

The isolates were screened for the presence of metallo β-lactamases (MBL) phenotypically in addition to amplification of genes by PCR. Both methods yielded negative results, suggesting no presence of MBL in any of the isolates.

Chapter 4: Discussion

4.1 Diversity in *bla*$_{\text{OXA-51-like}}$ genes

bla$_{\text{OXA-51-like}}$ genes are ubiquitous in *A. baumannii* and are used for identification as well as typing-scheme in assigning isolates to particular sequence groups (Hamouda *et al.*, 2010)

In the current study a large diversity was found in the sequences of *bla*$_{\text{OXA-51-like}}$ with eight different gene families identified. This is particularly interesting given the short duration of isolate collection (1.5 years) as well as the isolates deriving from only two hospitals.

Previous reports of the genetic diversity of *A. baumannii* isolates showed the presence of seven different *bla*$_{\text{OXA-51-like}}$ genes collected from 200 isolates between 1982 and 2005 (Merkier & Centrón, 2006). The current study hence gives an insight into the diversity of isolates present in Egyptian hospitals.

Three large groups around OXA-66, OXA-69 and OXA-98 were identified by *Evans et al.* (2008) in addition to other unrelated branched enzymes (Figure 46). European Clones (EC), now known as Worldwide clones 1 and 2, are successful lineages reported in outbreaks from around the world and associated with OXA-69 and OXA-66 clusters respectively (reviewed by Zander *et al.* 2012; Evans *et al.* 2008). Evans *et al*. (2008) identified OXA-65 as a central hub and ancestral to all other OXA-51-like enzymes and that there was a clear pathway to the OXA-69 and OXA-66 clusters.

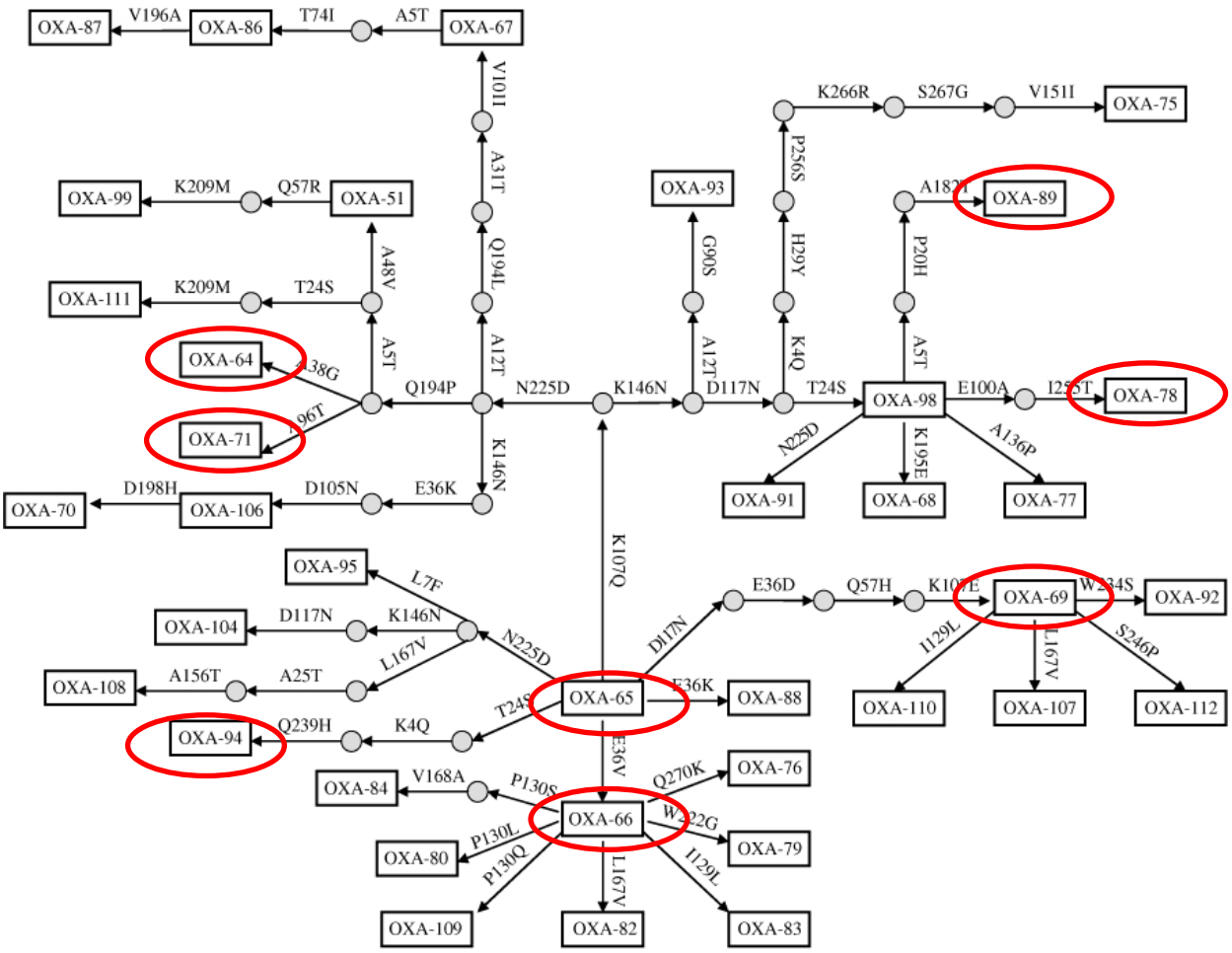


Figure 46: OXA-51-like enzyme linkage map with the enzymes identified in this study circled in red. From Evans *et al* (2008)**.**

As seen in figure 46, the *bla*$_{\text{OXA-51-like}}$ genes identified in the current study are widely scattered in the linkage map and not clustered around the same group. This indicates a diverse origin on the isolates.

The majority of isolates in the current study belonged to *bla*$_{\text{OXA-65}}$ (n=14), which shows the presence of the ancestral *bla*$_{\text{OXA-51-like}}$ gene in *A. baumannii* in Egypt. This may also explain the

large diversity found in the current collection was a result of evolution of the ancestral gene, rather than the foreign carriage of clones into the country.

bla$_{\text{OXA-94}}$, forms a branch of *bla*$_{\text{OXA-65}}$ cluster with three amino acid substitutions in the resultant protein. Two isolates from the NCI were recovered one day apart, both harbouring *bla*$_{\text{OXA-94.}}$ The NCI is an old hospital in Cairo, built in the early 1970s. If we assume that *A. baumannii* has been present in the NCI since it was built, *bla*$_{\text{OXA-94}}$ might have evolved from *bla*$_{\text{OXA-65.}}$ This can however only be determined if a study is performed on all *A. baumannii* isolates since the establishment of the NCI.

The OXA-66 enzyme cluster is the largest group identified by Evans *et al* (2008) with seven different groups originating from it. *bla*$_{\text{OXA-69}}$, *bla*$_{\text{OXA-66}}$ and *bla*$_{\text{OXA-71}}$ have been associated with Worldwide (WW) Clones I, II and III respectively and all have been identified in the current study (Zarrilli *et al*. 2009; Evans *et al*. 2008; Seifert *et al*. 2005; Zander *et al*. 2012). WW1 and WW2 seem to be more geographically widespread than other clones (Higgins *et al*. 2010). *bla*$_{\text{OXA-66}}$ and *bla*$_{\text{OXA-71}}$ genes were identified in both hospitals which may indicate local distribution in Egyptian hospitals. *bla*$_{\text{OXA-69}}$, on the other hand, was found in two isolates in the ICU outbreak in early 2011 at CCH only. This illustrates the extent of spread of the major lineages of *A. baumannii*. Furthermore, the presence of the cluster enzymes OXA-66 and OXA-69 shows the presence of the major lineages of *A. baumannii* in Egypt.

The OXA-98 cluster contains five related enzymes: OXA-89 with three amino acid substitutions, OXA-78 with two amino acid substitutions, OXA-68, OXA-77 and OXA-91 with one amino acid substitution. *bla*$_{\text{OXA-89}}$ and *bla*$_{\text{OXA-78}}$ were both identified in single isolates from CCH. It cannot be determined if the mutations from *bla*$_{\text{OXA-98}}$ happened in Egypt to generate these

enzymes. Reports of *A. baumannii* encoding OXA-78 and OXA-89 from as early as the 1980s (Evans *et al*. 2008) in Argentina, suggest that the presence of these enzymes occurred before the identification of *A. baumannii* as a major hospital pathogen (Evans *et al*. 2008).

bla$_{\text{OXA-71}}$ and *bla*$_{\text{OXA-64}}$ belong to branches rather than belonging to a group. Both enzymes are present in both hospitals. *bla*$_{\text{OXA-64}}$ is now repeatedly reported in isolates from the Middle East (Opazo *et al*. 2012a; Al-Hasan 2012). *bla*$_{\text{OXA-71}}$ is closely related to *bla*$_{\text{OXA-64}}$ as they seem to originate from a common ancestor, but are part of different clones: WW3 and WW7, respectively (Figure 46) (Zander *et al*. 2012).

The current results suggest sporadic cases of *A. baumannii* rather than the presence of a single clone in the hospitals.

WW1 and WW2 contain the largest group of *bla*$_{\text{OXA-51}}$ variants, which according to (Zander *et al*. 2012) might indicate relatively older lineages. Furthermore, carbapenem therapy may also play an important role in the selection of successful lineages of OXA-51, especially when it is the sole carbapenem resistance mechanism and associated with IS*Aba1*.

4.1.1 Insertion of IS*Aba2* upstream of *bla*$_{\text{OXA-89}}$

bla$_{\text{OXA-51-like}}$ genes associated with the presence of IS*Aba1* upstream in conferring resistance to carbapenems (reviewed by Peleg *et al*. 2008). IS*aba9* has also been reported upstream of *bla*$_{\text{OXA-51}}$ in a carbapenem resistant *A. baumannii* (Figueiredo *et al*., 2009). IS*Aba2* has been found upstream of *bla*$_{\text{OXA-58}}$ serving to mobilize the gene as well as providing promoter for the expression of resistance (Poirel & Nordmann, 2006b).

In isolate 22055 from CCH, IS*Aba2* was identified upstream of *bla*$_{\text{OXA-89.}}$ The isolate was resistant to carbapenems (MIC 128mg/L for imipenem and 256mg/L for meropenem). With no other resistance mechanism identified, the resistance was attributed to the localization of IS*Aba2* upstream of *bla*$_{\text{OXA-89/100}}$ and providing a suitable promoter. This is the first report of IS*Aba2* upstream of a *bla*$_{\text{OXA-51-like}}$ gene.

4.1.2 Epidemiological relatedness of *bla*$_{\text{OXA-51-like}}$ genes by PFGE

The diversity of *bla*$_{\text{OXA-51-like}}$ genes was further illustrated in their PFGE pattern. The overall similarity at 28% suggested the presence of different clones within the same hospital. This could be due to the localization of the patients in different wards and at different times in the hospital. Even for isolates recovered from the ICU at different times, there seems to be significant variability in profiles.

The distribution of *bla*$_{\text{OXA-51-like}}$ genes in the different governorates was assessed aiming to explain the observed diversity. *bla*$_{\text{OXA-65}}$ gene, the most prevalent gene identified, was found in patients from diverse origins from eight different governorates. *bla*$_{\text{OXA-64}}$ was present in patients from four governorates. *bla*$_{\text{OXA-66}}$ seems more localized in patients from one governorate, and similarly for *bla*$_{\text{OXA-69}}$. All other genes are found in a single isolate, and would therefore not give an accurate result.

If we assume that the different clones were brought by patients into the hospital we can therefore see the wide distribution of clones in the different regions in Egypt. Additionally, since *bla*$_{\text{OXA-65}}$ is thought to be ancestral to all *bla*$_{\text{OXA-51-like}}$ genes (Evans *et al*., 2008), the prevalence of this enzyme in many governorates may reflect the evolution of the different genes in bacteria in the

different regions of Egypt. Furthermore, five worldwide clones are present in the two Egyptian hospitals: WW1, WW2, WW3, WW5, WW7 (Zander *et al*., 2012).

If this hypothesis is to be taken further, two patients from Qina, a governorate in Upper-Egypt were found to harbour $bla_{\text{OXA-64}}$ and $bla_{\text{OXA-71}}$ which are closely related enzymes (Evans *et al*. 2008). These two isolates might have evolved from the same origin and got disseminated in the environment and was later brought by the patients to CCH.

The linkage map below shows the relationship of enzymes identified in the different Egyptian governorates. (Figure 47)

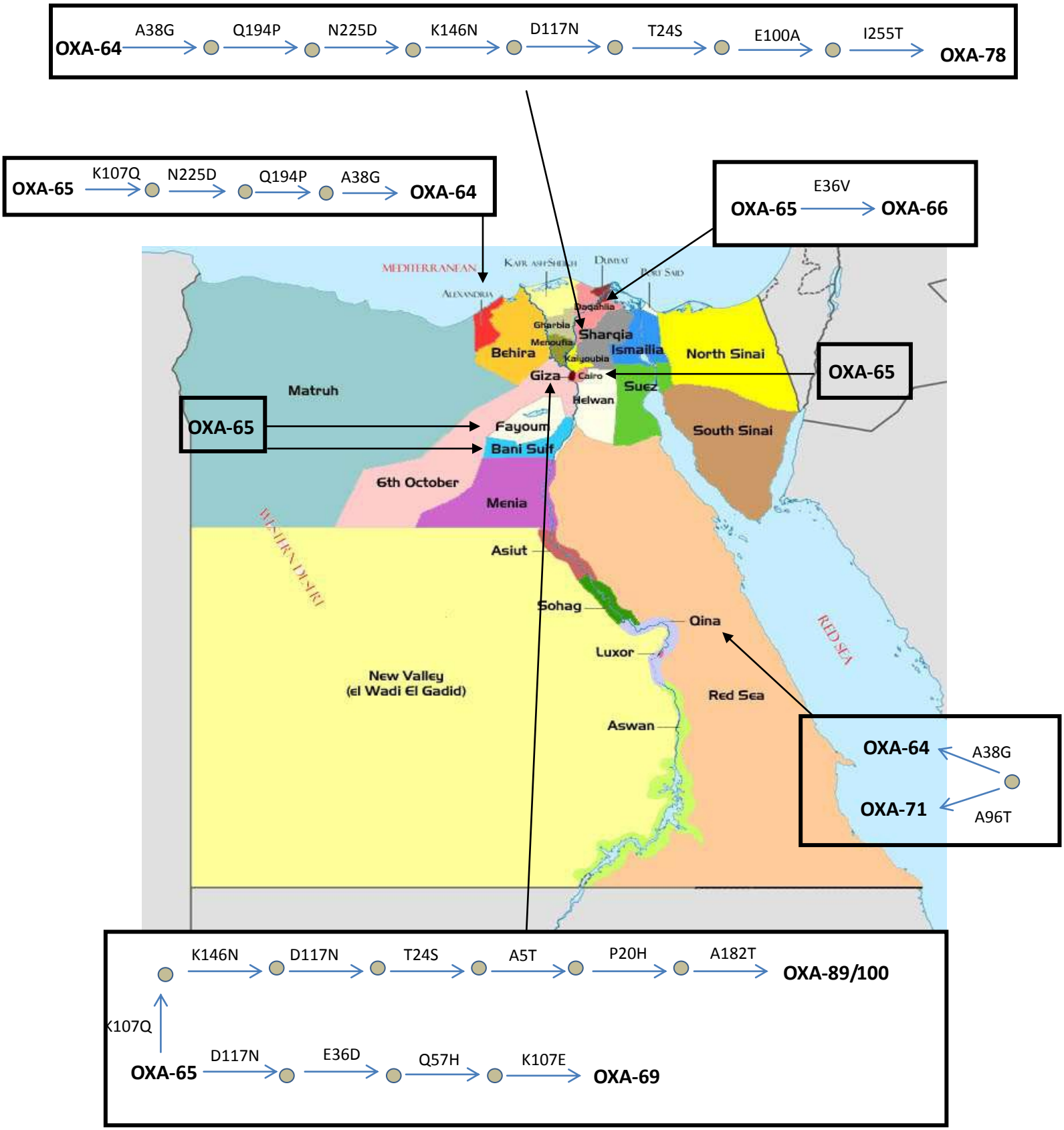


Figure 47: Enzyme Linkage map of OXA-51 like enzymes in governorates

4.2 Multi-locus sequence typing

A total of 10 isolates were chosen for analysis by MLST. The 10 isolates were representative of each *bla*$_{\text{OXA-51-like}}$ gene identified, and when the same gene was identified in both hospitals, an isolate from each hospital was randomly selected for comparison. Table 25 shows the isolates chosen for MLST, their *bla*$_{\text{OXA-51-like}}$ gene and the designated ST.

ISOLATE	HOSPITAL	*bla*OXA-51-like GENE	ST
8357	CCH	64	408
9925-Sam	NCI	64	409
1780	CCH	65	410
634	CCH	69	108
P67 - Azak	NCI	64	411
21174	CCH	78	412
22055	CCH	100	413
161	CCH	71	414
P38 - Ysf	NCI	94	331
14611	CCH	66	208

Table 25: List of *bla*$_{\text{OXA-51-like}}$ gene associated with the different STs.

Given the diversity of the isolates in this study, it was expected to find novel STs. In fact, of the 10 isolates typed by MLST, seven novel STs for isolates: 8357, 9925-SAM, 1780, P67-AZak, 21174, 22055, 161 and 14611 were assigned ST408 – ST414. Isolates 634, P38-Ysf and 14611 were assigned ST108, ST331 and ST208, respectively. These STs have previously been identified in Argentina and Brazil and USA (www.pubmlst.org/abaumannii last accessed 14/12/2012).

Isolates 9925-SAM and P67-AZak both from the NCI harboured the same *bla*$_{\text{OXA-51-like}}$ gene, were assessed for their similarity, and the prevalence of STs in the same hospital. Interestingly, these two isolates were assigned two novel STs: ST409 for SAM and ST411 for P67-AZak. As seen in table 21 (results section), ST409 and ST411 share, however, four similar alleles: *gyrB, gdhB, recA and rpoD*. This similarity shows a degree of relatedness in those strains.

The different STs identified in this study further illustrate the large diversity found in the collection of strains. This could indicate the presence of distinct clones in the two hospitals, especially that they were isolated at different months and in separate wards. Additionally, this diversity may also represent dissimilar clones brought to the hospitals from diverse geographical locations. To determine the prevalence of a certain ST in Egyptian hospitals, a larger study should be done on more Egyptian hospitals in diverse geographical locations.

Furthermore, no evolutionary relationship or clonal complexes can be inferred for the isolates in this study using e-BURST, endorsing the diversity of the strain collection. A population snapshot represented no linkage between the STs (figure 22). However, the population snapshot showed the relatedness to other STs identified from around the world. ST208, for example, is ancestral to several STs including ST89, ST88, ST190, ST225, ST75 identified in different parts of the world such as Japan, China, Thailand, Korea, Italy, Australia, Portugal and the Czech Republic. ST108 has been identified in Argentina and Brazil an shared six alleles with ST109 from Argentina, and which also seemed to be the ancestral ST in its group. ST419, also sharing six alleles with ST108, decended from ST108, according to the population snapshot (figure 22).

MLST is useful in global epidemiological studies particularly due to the transferability of typing data between different laboratories resulting in an easier comparison of results (Hamouda *et al.*

2010; Bartual *et al.* 2005; Wisplinghoff *et al.* 2008). The given results lead us to speculate about the efficiency of using MLST as a typing method for sporadic cases such as those represented in this study. A larger population of isolates, preferably from different hospitals in Egypt may be better represented by MLST to determine the prevalent ST as well as the evolutionary relationship of the isolates and their relatedness.

Previous reports of the different typing methods have shown a better correlation between sequencing of $bla_{\text{OXA-51-like}}$ and MLST than with PFGE (Hamouda *et al.* 2010) . In this study, as the isolates are sporadic, MLST was not correlated with $bla_{\text{OXA-51-like}}$ genes, as seen in the case of P67-AZak, 9925-SAM and 8357, all harbouring $bla_{\text{OXA-64}}$ but assigned different STs.

The PFGE profiles of isolates typed by MLST further illustrate the diversity, showing the distinct patterns. Thus, this endorses the hypothesis of the isolates being brought into the centres by different patients and from distinct geographical locations around Egypt.

4.3 The contribution of acquired class D β-lactmases to carbapenem resistance

The NCCN guidelines for treatment of Febrile Neutropenia are followed by physicians in both the National Cancer Institute and The Children's Cancer Hospital in Cairo. First line antibiotics are a broad-spectrum plus an anti-pseudomonal agents. Changing the antibiotics occur either according to the blood culture susceptibility profile or if the patient condition is not improving after three days of antibiotics administration. The second line of treatment are carbapenems in proven Gram-negative infections. Colistin is not available in most Egyptian hospitals, which is the reason behind the extensive use of carbapenems to treat severe *A. baumannii* infections, sometimes even despite resistance. Imipenem and Meropenem are the only carbapenems available in Egyptian hospitals.

Seventy-three percent of the isolates were resistant to carbapenems, and this is associated with all three CHDL genes found in this study.

4.3.1 *bla*$_{\text{OXA-23}}$

bla$_{\text{OXA-23}}$ was the most common gene identified in the isolates, accounting for 53%. Three genetic structures were determined to harbour and mobilize *bla*$_{\text{OXA-23:}}$ Tn*2006*, Tn*2007*, and Tn*2008* (Mugnier *et al*. 2010; Wang *et al*. 2011) . IS*Aba1* was only found upstream of *bla*$_{\text{OXA-23}}$ in the current study, which indicates the mobilization of the gene by the presence of the IS element, showing a structure of a truncated transposon. . This is consistent with the observed resistance to carbapenems, provided by the promoters in IS*Aba1* upstream of the gene.

bla$_{\text{OXA-23}}$ has been reported in different regions of the Middle East, in strains from The United Arab Emirates, Algeria, Libya and Bahrain and the associated genetic structures were diverse: Tn*2008*, Tn*2006*, Tn*2007* as well as IS*Aba1* (Mugnier *et al.* 2010; Mugnier *et al*. 2008; Mugnier *et al*. 2009). Interestingly, an isolate from Egypt harbouring *bla*$_{\text{OXA-23}}$ was found to be plasmid-mediated and associated with IS*Aba1* as its genetic structure (Mugnier *et al*. 2010). The given results might indicate the prevalence of this genetic environment of *bla*$_{\text{OXA-23}}$ in Egyptian isolates.

According to the PFGE pattern (figure 25), four epidemiologically related groups can be seen, sharing >80% similarity. Isolates SF-461 and 4842 were from two different hospitals but shared similar *bla*$_{\text{OXA-51-like}}$ genes, and a similar situation was found for isolates P391-AH and 10262.

PFGE profiles of the isolates are also very diverse which shows the dissemination of *bla*$_{\text{OXA-23}}$ in different *A. baumannii* clones. The ICU outbreak in CCH belonged to two clones, as revealed by their PFGE profiles and sequencing of the instrinsic *bla*$_{\text{OXA-51-like,}}$ but the carbapenem resistance was mediated by *bla*$_{\text{OXA-23,}}$ detected in all isolates in the outbreak.

bla$_{\text{OXA-23}}$ was detected in different clones with different *bla*$_{\text{OXA-51-like}}$ genes. The majority of *bla*$_{\text{OXA-23}}$ genes were present in isolates harbouring *bla*$_{\text{OXA-65}}$ (10 out of 18 isolates). This association with the ancestral *bla*$_{\text{OXA-51-like}}$ gene further shows the prevalence of *bla*$_{\text{OXA-23}}$ in Egypt.

4.3.2 *bla*$_{OXA-58}$

Five isolates were positive for *bla*$_{OXA-58}$ genes in this study, all being resistant to imipenem and meropenem, with the exception of isolate 14298 which was intermediate to meropenem. IS*Aba3* was detected flanking *bla*$_{OXA-58}$ genes in all isolates. Two isolates: 14298 and P67-AZak harboured an interrupted IS*Aba3* upstream, and sequencing revealed the presence of IS*6* family sequences: IS*1006* and IS*1008* in those strains.

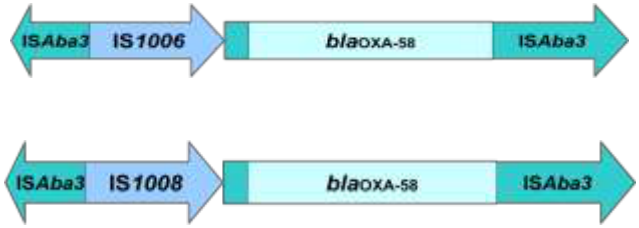


Figure 48: Schematic representation of the genetic environment of *bla*$_{OXA58}$

bla$_{OXA-58}$ has been frequently identified in carbapenem-resistant *A. baumannii* from different regions of the world (Coelho *et al*. 2006; Poirel & Nordmann,2006). IS*Aba3* was found flanking the gene and hence served as a promoter upstream in addition to effective mobilization of *bla*$_{OXA-58}$ (Chen *et al*. 2008). Hybrid promoters, with an interrupted IS*Aba3* sequence upstream, have also been identified to confer high level of carbapenem resistance by enhancing the transcription of *bla*$_{OXA-58}$ (Chen *et al.* 2010). Other ISs including IS*Aba1* and IS*Aba2* have also been identified in providing suitable promoters for *bla*$_{OXA-58}$ in *A. baumannii* (Poirel & Nordmann, 2006)

This thesis shows that, although the upstream IS*Aba3* in both strains was interrupted by insertion sequences, the promoters were not hybrid, as the -35 and -10 sequences were solely located in IS*1006* and IS*1008*. Additionally, the IS*Aba3* was truncated upstream of *bla*$_{OXA-58}$ gene by the

transposition of IS*1006* and IS*1008*, which hence illustrates that IS*1006* and IS*1008* inserted upstream of $bla_{\text{OXA-58,}}$ and provided promoter sequences for effective transcription of the gene. The localization of IS*1006* and IS*1008* disrupted the promoter provided by IS*Aba3.*

IS*1006* has only been characterized in *Acinetobacter nosocomialis* (Chen *et al*., 2010), so the current finding is the first report of this IS in *A. baumannii*. The plasmid containing bla_{OXA58} and the associated upstream environment was probably transferred to *A. baumannii* by horizontal gene transfer. In the study by Chen *et al* (2010), IS*1006* was inserted into the IS*Aba3* sequence rather than interrupting it, thus explaining the presence of a hybrid promoter. The MIC of imipenem was 16mg/L (Chen *et al*., 2010), whereas in isolate 14298, the MIC was 8mg/L and it was intermediate to Meropenem (MIC 2mg/L). This may indicate that IS*1006* on its own does not provide a strong promoter, when compared to the resistance observed in the isolate harbouring a hybrid promoter.

IS*1008* also reported by Chen *et al.* (2006), was detected on a plasmid of a carbapenem-resistant *A. baumannii* isolate. In contrast to IS*1006*, the IS*Aba3* upstream of $bla_{\text{OXA-58}}$ was truncated by the insertion of IS*1008*. Hence, this is similar to isolate P67-AZak in the current study. On the other hand, the promoter in the report by Chen *et al*. (2006) was hybrid with -10 box located in the IS*Aba3* and -35 box located in the IS*1008*, whereas for isolate P67-AZak they were both located in IS*1008.* Interestingly, the MICs of imipenem and meropenem (64mg/L and 16 mg/L, respectively) reported by Chen *et al* (2006) were identical to those of isolate P67-AZak in the current study. The given results show that IS*1008* alone can provide a suitable strong promoter for high-level resistance to carbapenems.

Although both *IS1008* and IS*1006* both belong to the same family IS*6*, they seem to differ in their contribution to carbapenem resistance. Their localization upstream of *bla*$_{\text{OXA-58}}$ serves to provide promoter sequences for transcription of the gene by interrupting the upstream IS*Aba3* sequence. The promoter provided by IS*1008* was stronger as a higher MIC was seen for imipenem and meropenem when compared to the isolate harbouring IS*1006*, which was only resistant to imipenem and intermediate to meropenem.

IS*6* family sequences act as effective promoters for the expression of carbapenem resistance by *bla*$_{\text{OXA-58.}}$ In the current study, IS*1006* and IS*1008* were found to interrupt the IS*Aba3* sequence upstream and hence they alone provided the promoters. Additionally, the similarity in results between the current study to those reported by Chen *et al*. (2006, 2010) illustrates how similar genetic environments can be found in distinct *A. baumannii* isolates from diverse parts of the world.

4.3.3 *bla*$_{\text{OXA-40}}$

One carbapenem-resistant isolate was found positive for *bla*$_{\text{OXA40.}}$ Sequence alignment showed no silent mutations in the sequence, with 100% identity to OXA-40 sequence described by Bou *et al*. (2000). No plasmid was detected in that isolate, suggesting a chromosomal localization of the gene. *bla*$_{\text{OXA-40}}$ has been identified in different parts if the world, including Spain, USA and Bahrain (reviewed by Peleg *et al.* 2008; Mugnier *et al*. 2009). Localization can be chromosomal or plasmid-mediated, and is not associated with any insertion sequence (Peleg *et al.* 2008; Castanheira *et al.* 2008). *bla*$_{\text{OXA40}}$ appear to produce high MICs of imipenem and meropenem, even without an IS element, and inactivation of this gene leads to sensitivity to carbapenems

(reviewed by Peleg *et al*. 2008; Héritier *et al*. 2005). The MICs for isolate 14611 harbouring *bla*$_{OXA40}$ was 128mg/L for imipenem and 32mg/L for meropenem, revealing the effect of this gene on carbapenem resistance. These MICs are amongst the highest seen in the isolates, suggesting that *bla*$_{OXA-40}$ contribute to produce higher MICs to carbapenems when compared with MIC for isolates with *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ genes (reviewed by Peleg *et al*. 2008; Héritier *et al*. 2005).

bla$_{OXA24}$ was reported in a plasmid isolated from *A. calcoaceticus* (Merino *et al*. 2010). The full plasmid was sequenced and revealed that the *bla*$_{OXA24}$ gene was flanked by XerC/XerD-like sites, responsible for mobilization of the gene. This structure was not detected in the current study, which may explain the limited spread of this gene in the isolates, particularly as it was not localized on a plasmid. The localization of *bla*$_{OXA-40}$ in the chromosome, in addition to the lack of associated insertion sequences largely contributes to the limited distribution not just in the Egyptian isolates, but around the world, with fewer reports of outbreaks caused by *bla*$_{OXA-40}$ than *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ (reviewed by Peleg *et al*. 2008).

4.4 The contribution of Extended-spectrum β-lactamases to ceftazidime resistance

Extended-spectrum β-lactamases (ESBLs) are increasingly reported in *A. baumannii*. They contribute to resistance toward broad-spectrum cephalosporins such as ceftazidime, cefotaxime and ceftriaxone and are inhibited by β-lactamase inhibitors such as tazobactam, clavulanic acid, sulbactam (Poirel *et al*. 2012). *bla*$_{\text{PER-like}}$, *bla*$_{\text{VEB}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{GES}}$ genes have been reported in clinical *A. baumannii* infections worldwide (reviewed by Peleg *et al*. 2008).

In the current study, three variants of *bla*$_{\text{PER-like}}$ genes were identified in three strains: *bla*$_{\text{PER-1,}}$ *bla*$_{\text{PER-3}}$, and *bla*$_{\text{PER-7}}$, as well as the identification of *bla*$_{\text{TEM-1}}$ in 6 isolates.

4.4.1 *bla*$_{\text{PER-1}}$

bla$_{\text{PER-1}}$ was detected in isolate 2625 from CCH. The associated genetic environment was determined to consist of two insertion sequences: IS*Pa12* and IS*Pa13* flanking *bla*$_{\text{PER-1.}}$

bla$_{\text{PER-1}}$ was initially identified in *Pseudomonas aeruginosa* and later in several bacterial species including *A. baumannii* (reviewed by Peleg *et al*., 2008; Poirel *et al*., 2005; Bonnin *et al*., 2011). The localization of *bla*$_{\text{PER-1}}$ gene can either be on plasmids or chromosome with an associated upstream IS enhancing its expression (reviewed by Peleg *et al*., 2008; Poirel *et al.*, 2005a; Poirel *et al*., 2012). The gene was not located in a class 1 integron, but rather associated with a transposon-related structure: Tn*1213* (Poirel *et al*., 2005a).

Poirel *et al.* (2005) identified Tn*1213* always localized inside an IS*Pa*14 element, which is similar to IS*Aba3* in *A. baumannii*, and this may suggest that the acquisition of the genetic environment of *bla*$_{\text{PER-1}}$ initially occurred in *A. baumannii* rather than *P. aeruginosa.*

No plasmids harbouring *bla*$_{PER-1}$ were detected in the current isolates, which may explain the limited dissemination of *bla*$_{PER-1}$. Isolate 2625 harboured *bla*$_{PER-1}$ which represented a sporadic case of PER-1 enzyme in the hospital.

Additionally, the genetic environment of *bla*$_{PER-1}$ seems to be conserved in isolates from distinct geographical locations, with the mobilization and expression of the gene mediated by the presence of insertion sequences. PER-1 is widely distributed in the Mediterranean region, with several reports in Greece and Turkey. In fact, a study conducted in Turkey found 32% of *A. baumannii* harbouring *bla*$_{PER-1}$ as the resistance mechanism against ceftazidime (Poirel *et al*. 2012). A case report of a Hungarian tourist, hospitalized in Egypt was found to harbour a PER-1 producing *A. baumannii* strain. Hence, this means that PER-1 is present in other Egyptian hospitals, but maybe due to the lack of reports, we cannot get an accurate estimation of the prevalence PER-1 in Egypt.

4.4.2 *bla*$_{PER-3}$

This is the first identification of *bla*$_{PER-3}$ in a clinical *A. baumannii* isolate, resistant to all antibiotics except carbapenems. Previous reports of this gene have been from *Aeromonas punctata* isolate from France and recently also in Taiwan. In that strain, *bla*$_{PER-3}$ was reported to be localized on both plasmids and chromosome (Wu *et al*. 2011; Toleman *et al*. 2006); however, in this study, it was identified on the chromosome alone. The genetic environment upstream of *bla*$_{PER-3}$ was similar to the *A. punctata* isolate identified in France (GenBank accession no. AY740681.1), being located in a complex-class-1 integron.

The mechanism of acquisition of the gene by *A. baumannii* is unknown, but I hypothesize that the *bla*$_{PER3}$ gene was acquired by *A. baumannii* through horizontal gene transfer from *A. punctata*. The genetic transfer of *bla*$_{PER-3}$ is also facilitated by the localization of the gene within an integron, which can easily be transferred between organisms. Much of the increasing incidence of resistance around the world has been attributed to the localization of resistance genes within mobile genetic elements (Toleman *et al*. 2006a). *bla*$_{PER-3}$ might have been transferred to *A. baumannii* by a plasmid harbouring the integron, which later migrated into the chromosome.

The structure of PER-1 has been studied by Trainer *et al* (2000) and revealed a completely new fold of the Ω-loop region, organised in secondary structure elements, compared with other ESBLs. PER-3 is a point-mutant derivative of PER-1 (Wu *et al*. 2011) harbouring the following change H134L. According to the structure determined by Trainer *et al* (2000), this mutation is positioned in H4 helix of the secondary structure of the Ω-loop region. The Ω-loop region is associated with the active site of class A β-lactamases (Banerjee *et al.* 1998), and the observed mutation could explain the reduced resistance to ceftazidime, MIC = 64 mg/L, in the isolate harbouring *bla*$_{PER-3}$ when compared with other *A. baumannii* isolates harbouring *bla*$_{PER-1}$ and *bla*$_{PER-7,}$ MIC >256 mg/L and 256 mg/L, respectively.

Although *bla*$_{PER-3}$ shares considerable homology to *bla*$_{PER-1}$, the upstream regions of these genes differ significantly. *bla*$_{PER-3}$ lacks IS elements flanking the gene and harbours IS*CR1,* which is closely associated with class A ESBLs and facilitates the movement of these resistance genes (Toleman *et al*. 2006a)

4.4.3 *bla*$_{PER-7}$

bla$_{PER-7}$ was identified in a single isolate from the NCI, resistant to all antibiotics. The first identification of *bla*$_{PER-7}$ was in a clinical *A. baumannii* isolate from France (Bonnin *et al*. 2011) and later in an isolate from the United Arab Emirates (Opazo *et al*., 2012a). The localization of this gene can be chromosomal or plasmid-mediated (Bonnin *et al*. 2011; Opazo *et al*. 2012a). In the current isolate, *bla*$_{PER-7}$ was located on a ~155kb plasmid, smaller than the 200kb plasmid identified harbouring the same gene in the United Arab Emirates isolate (Opazo *et al*. 2012a).

Interestingly, many similarities are shared between the current isolate and those reported in France and UAE. All three isolates contained the *bla*$_{OXA-64}$ intrinsic gene, as well as harbouring the acquired *bla*$_{OXA-23}$. IS*Aba1* was present upstream of *bla*$_{OXA-23}$ in the current isolate as well as the one reported in UAE. The genetic structure of *bla*$_{PER-7}$ was similar in the three strains, being located within a complex class-1 integron. orf*513* is a putative transposase associated with several antibiotic resistance genes (Toleman *et al*. 2006b) and it was found directly upstream of *bla*$_{PER-7}$ hence mediating the expression of the resistance gene. orf*513* is also associated with class-1 integron structures (Toleman *et al*. 2006b; Bonnin *et al*. 2011).

A novel ST, ST409, was assigned for the isolate harbouring *bla*$_{PER-7}$ in the current study. The isolate from UAE was also typed by MLST and assigned ST110 (Opazo *et al,* unpublished results). ST409 and ST110 are closely related and only differ at two loci. This indicates that both isolates may share a similar origin.

Given that the strains in UAE and France were isolated in 2008 and 2010, respectively, plasmid-mediated PER-7 originated in the UAE, and later migrated to the chromosome as seen in the

French isolate (Opazo *et al*., 2012a). The results further suggest the presence of the plasmid containing PER-7 in the Middle East.

4.4.4 *bla*$_{TEM}$

TEM-like enzymes comprise one the largest group of ESBLs identified in the clinical setting, comprising 196 variants (Poirel *et al*. 2012). TEM-like enzymes are mainly plasmid mediated and frequently associated with *Enterobactericea*. To date, more than 130 variants of TEM-like enzymes have been reported (http://www.lahey.org/Studies/ last accessed 5 December 2012). In *A. baumannii*, only TEM-92 and -116 have been identified in Europe to cause resistance to cephalosporins (Peleg *et al*. 2008).

In this thesis, *bla*$_{TEM-1}$ was identified in 6 isolates, 5 of which are from CCH. TEM-1 enzymes are penicillinases, with a narrow-spectrum ESBL activity. This enzyme is prevalent in *A. baumannii* and has low-level ceftazidime hydrolyzing activity (Poirel *et al*. 2012; Peleg *et al*. 2008; Bush 2001; Livermore & Woodford 2006). TEM-like variants have developed to hydrolyse third-generation cephalosporins more efficiently and hence cause resistance.

Furthermore, TEM-1 was identified in 40% of isolates in Walter Reed Army Medical Centre, obtained from soldiers injured in Iraq and Afghanistan (Hujer *et al.* 2006).

In the current study, the detection of a narrow-spectrum β-lactamase TEM-1 does not serve as a threat to the spread of resistance, but can create a potential problem if mutations occur in the gene, giving rise to enzymes with ceftazidime-hydrolysing activity such as TEM-96. The

plasticity of ESBLs structurally and functionally, facilitates evolutionary processes to give rise to a large variety of enzymes with expanded substrate spectrum (Gniadkowski, 2008).

4.5 Acinetobacter-derived cephalosporinases (ADC)

bla$_{ADC}$ are chromosomally encoded genes inherent to *A. baumannii* (reviewed by Peleg *et al.* 2008; Segal *et al.* 2004). Resistance to ceftazidime and extended-spectrum cephalosporins is only mediated when an IS is present upstream of *bla*$_{ADC}$ gene, thereby upregulating the expression. IS*Aba1* is the most commonly associated IS element with ADC (Segal *et al*. 2004; Corvec *et al.* 2003; Mugnier *et al.* 2009). IS*Aba125* has also been identified upstream of *bla*$_{ADC}$ genes and contributed to ceftazidime resistance (Lopes *et al*., 2011. P601, ECCMID).

bla$_{ADC}$ was detected in all the strains in the current strains, with 6 isolates harbouring IS*Aba1* upstream of *bla*$_{ADC}$ gene and contributing to an MIC of ceftazidime of 256mg/L. ADC β-lactamases are only capable of hydrolysing extended-spectrum cephalosporins when it is 'overproduced'.

The results give insight into the importance of IS*Aba1* in the of *A. baumannii* genome. It plays a vital role in expression, acquisition and mobilization of several resistance genes, such as *bla*$_{OXA-51-like}$, *bla*$_{OXA-23}$ and *bla*$_{ADC}$ (Héritier *et al*. 2006). IS*Aba1* seems to enhance the plasticity of the *A. baumannii* genome. It is widely distributed in *A. baumannii* worldwide, present in both susceptible and resistant isolates (Mugnier *et al*. 2009). IS*Aba1* is capable of transposition and consequently inserts upstream of genetic genes when a selective advantage is present (Turton *et al*. 2006).

Chapter 5: Conclusions:

A variety of resistance mechanisms are present in the isolates obtained from the two centres in Egypt. Additionally, several clones are present which harbour and express these resistance mechanisms. Unlike most hospitals, there were several clones spreading through and a number of sporadic strains as well. The diversity in *bla*$_{\text{OXA-51-like}}$ genes is seen in the isolation of 8 different genes in thirty-four isolates from two hospitals. The PFGE pattern also distinguishes the isolates into different groups and does not correlate with *bla*$_{\text{OXA-51-like}}$, suggesting the presence of epidemiologically diverse clones. Typing by MLST further illustrates the diversity of strains, with 8 different STs assigned, and the identification of the 10 isolates as singletons rather than belonging to a group.

The resistance to carbapenems is largely mediated by the expression of acquired carbapenemases: *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$ and *bla*$_{\text{OXA-58.}}$ *bla*$_{\text{OXA-23}}$ was the most commonly identified gene in the current study and it contributed to carbapenem resistance which was facilitated by IS*Aba1* located directly upstream of the gene. *bla*$_{\text{OXA-24}}$ was identified in a single isolate, probably located on the chromosome. *bla*$_{\text{OXA-58}}$ was identified in six isolates from both hospitals all flanked by IS*Aba3* element. Two isolate, however had IS*6* family ISs present upstream of *bla*$_{\text{OXA-58}}$, thereby interrupting IS*Aba3*. IS*Aba2* was identified upstream of *bla*$_{\text{OXA-89}}$, contributing to high-level resistance to carbapenems. This serves to further illustrate the role of insertion sequences in mediating resistance in *A. baumannii*.

The role of IS*Aba1* in expression of resistance genes is not only present in the acquired *bla*$_{\text{OXA-23}}$ gene, but also in its association with *bla*$_{\text{ADC.}}$ The presence of IS*Aba1* upstream of *bla*$_{\text{ADC}}$ correlated with high-level ceftazidime resistance.

Ceftazidime resistance in three other isolates was attributed to the presence of *bla*$_{\text{PER-like}}$ genes in those isolates. The first identification of *bla*$_{\text{PER-3}}$ in a clinical *A. baumannii* isolate serves to illustrate the genetic plasticity in the acquisition of novel resistance genes from other organisms.

The heterogeneity of resistance mechanisms present in this study is very unique. It shows how different resistance mechanisms act together in response to the environment and the selective pressures undertaken by the usage of antibiotics, as well as more importantly, it highlights the role of evolution of *A. baumannnii* genome and the presence of different clones within the same hospital in a relatively short time. The role of the different IS elements further enhances the expression of different resistance mechanisms and provide more plasticity in the genome.

Previous reports of the genetic diversity of *A. baumannii* isolates showed the presence of seven different *bla*$_{\text{OXA-51-like}}$ genes collected from 200 isolates between 1982 and 2005 (Merkier & Centrón 2006). The current study hence gives an insight into the diversity of isolates present in Egyptian hospitals.

More research is required to determine the prevalence of a clone in Egyptian hospitals. The diversity seen in the current study may be attributed to the centres being tertiary referral hospitals, where the isolates have consequently been brought to the hospitals by the patients from different regions in Egypt.

The lack of adequate infection control together with the lack of reporting and the limited funds available for research in developing countries all contribute to the lack of knowledge we have of the epidemiology of hospital acquired infection. There is an urgent need to fund and support research in developing countries, in order to prevent the clonal dissemination of antibiotic resistant organisms not only in the hospital setting, but in the community.

Antimicrobial stewardship programs aimed to preserve the activity of antibiotics and limiting the rates of resistance are strongly needed in developing countries like Egypt, as the inadequate usage of antibiotics in the community and hospitals all contribute to the rise in resistance, and promotes the organism to acquire and express novel resistance mechanisms.

A. baumannii is a very interesting pathogen due to its rapid development of resistance and the plasticity of its genome. Further research into the epidemiology of the organism, not only in Egyptian hospitals, but in other regions of the Middle East is one of my future research aims in order to get more information about the problem it serves.

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APPENDIX A

Published Papers

International Journal of Antimicrobial Agents xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Letter to the Editor

First report of *bla*$_{PER-3}$ in *Acinetobacter baumannii*

Sir,

Acinetobacter baumannii is an important opportunistic nosocomial pathogen with a remarkable ability to survive and disseminate. In addition, *A. baumannii* can acquire new antibiotic resistance genes and upregulate them, making it a problematic pathogen causing numerous outbreaks worldwide [1,2]. Patients at high risk for developing *A. baumannii* infections are those with prolonged hospitalisation and immune suppression, such as cancer patients [2]. Antibiotic selective pressure also contributes to the rising incidence of resistance [1].

The mechanisms of resistance in *A. baumannii* that cause most concern are the β-lactamases, and all the Ambler class enzymes (A–D) have been found in this species. Class D and class B β-lactamases mainly confer resistance to the carbapenems, whilst class A are mainly extended-spectrum β-lactamases (ESBLs) [1]. The first report of an ESBL in *A. baumannii* was *bla*$_{PER-1}$ in France [3], with PER-like variants in *A. baumannii* now increasingly reported worldwide. PER-3 was initially characterised from an *Aeromonas punctata* in France (GenBank accession no. AY740681.1) and recently in a medical centre in Taiwan [3], sharing 99% homology to *bla*$_{PER-1}$. Here we report the first identification of a *bla*$_{PER-3}$-producing *A. baumannii* isolate.

Isolate AB-15094 was obtained from a 9-year-old patient with osteosarcoma in 2010 at the Children's Cancer Hospital 57357 (Cairo, Egypt). Phenotypic identification was followed by sequencing of the *bla*$_{OXA-51-like}$ gene and restriction analysis of the 16S–23S rRNA spacer sequence [2,4].

Minimum inhibitory concentrations (MICs) were determined and interpreted according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines for imipenem, meropenem, ceftazidime and rifampicin. Detection of the OXA-type carbapenemases *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-51}$ and *bla*$_{OXA-58}$ as well as class A β-lactamase genes *bla*$_{PER-like}$, *bla*$_{VEB}$, *bla*$_{GES}$ and *bla*$_{TEM}$ and the class C β-lactamase gene *bla*$_{ADC}$ was done by standard PCR [3,4]. The class 1 integron harbouring the *bla*$_{PER-3}$ gene was analysed by standard PCR and sequencing methods [4]. *S1* nuclease digestion was performed to detect and size plasmids [3]. To investigate whether *bla*$_{PER-3}$ was plasmid-borne, a plasmid curing experiment was conducted. Susceptibility to ceftazidime was examined after curing and was compared with the initial susceptibility of the uncured strain.

Isolate AB-15094 was confirmed as *A. baumannii* harbouring the *bla*$_{OXA-65}$ gene. It was sensitive to imipenem and meropenem but resistant to ceftazidime and rifampicin, with MICs of 64 mg/L and 256 mg/L respectively. The isolate was positive for *bla*$_{PER-3}$ as revealed by sequencing. The strain was negative for all other ESBLs tested as well as for the acquired class D carbapenemases. No insertion sequence (IS) element was detected upstream of the *bla*$_{ADC}$ gene, suggesting no overexpression of the gene [4].

There was no hybridisation of *bla*$_{PER-3}$-specific probes with any of the isolate's three plasmids (60, 70 and 145.5 kb, respectively), suggesting chromosomal localisation of the gene. This was supported by the lack of change in ceftazidime susceptibility after 10 days of plasmid curing. Furthermore, attempted conjugal transfer was also negative. Sequencing upstream of *bla*$_{PER-3}$ revealed a complex class 1 integron structure harbouring *sul1*, *qacE*Δ1 in the variable 3'CS and ORF*513* directly upstream of the *bla*$_{PER-3}$ gene, serving as the transcriptional promoter. Fig. 1 shows the schematic representation of the genetic environment of *bla*$_{PER-3}$.

This isolate was obtained from a paediatric cancer patient in Egypt and is the first identification of *bla*$_{PER-3}$ in a clinical *A. baumannii* isolate. Previous reports of this gene have been from *A. punctata* in France and Taiwan where the *bla*$_{PER-3}$ gene was reported to be localised both on plasmids and on the chromosome [3]; however, in this study it was located on the chromosome alone. The genetic environment upstream of *bla*$_{PER-3}$ was similar to the *A. punctata* isolate identified in France (GenBank accession no. AY740681.1), being encompassed in a complex class 1 integron. The upstream regions of *bla*$_{PER-3}$ and *bla*$_{PER-1}$ differ significantly despite their structural homology. *bla*$_{PER-1}$ was part of transposon-related structure Tn*1213* consisting of IS*Pa12* and IS*Pa13* flanking the gene [1]. The *bla*$_{PER-3}$ in this study lacks these IS elements and harbours IS*CR1*, which is closely associated with class A ESBLs and facilitates the movement of these resistance genes [3,5].

PER-3 is a point-mutant derivative of PER-1 [3] harbouring the change Histidine134Leucine. This mutation is positioned in the H4 helix of the secondary structure of the Ω-loop region, which is associated with the active site of class A β-lactamases [5]. The observed mutation could explain the reduced resistance to ceftazidime (MIC = 64 mg/L) in isolate AB-15094 harbouring *bla*$_{PER-3}$ compared with other *A. baumannii* isolates harbouring *bla*$_{PER-1}$ and *bla*$_{PER-7}$, with MICs > 256 mg/L and 256 mg/L, respectively (unpublished results).

The mechanism of acquisition of *bla*$_{PER-3}$ by *A. baumannii* is unknown but is hypothesised to be mediated through horizontal

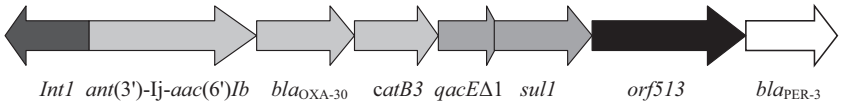

Fig. 1. Schematic diagram of the genetic environment of *bla*$_{PER-3}$. The genetic orientations are represented by horizontal arrows. The figure is to scale.

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http://dx.doi.org/10.1016/j.ijantimicag.2012.09.010

gene transfer from *A. punctata*. Genetic transfer of bla_{PER-3} is facilitated by localisation of the gene within an integron. bla_{PER-3} might have been transferred to *A. baumannii* by a plasmid harbouring the integron, which later migrated into the chromosome.

Nucleotide sequence accession no.

The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank nucleotide sequence database and has been assigned accession no. JX128136.

Funding: Self-funded.

Competing interests: None declared.

Ethical approval: Not required.

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28 June 2012

ORIGINAL ARTICLE 10.1111/j.1469-0691.2012.03979.x

IS*Aba825* controls the expression of the chromosomal $bla_{OXA-51-like}$ and the plasmid borne bla_{OXA-58} gene in clinical isolates of *Acinetobacter baumannii* isolated from the USA

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Abstract

Four non-repetitive, clonally related (ST114), carbapenem-resistant *Acinetobacter baumannii* strains isolated in the USA were examined to understand the mechanisms of carbapenem resistance including screening for the presence of an insertion sequence upstream of the $bla_{OXA-51-like}$ gene, which could be involved in the control and expression of the antibiotic-resistance gene. We observed that the main mechanisms of carbapenem resistance were the result of the over-expression of the $bla_{OXA-58-like}$ and the bla_{OXA-65} gene, both of which had the presence of IS*Aba825* upstream of the genes. The importance of this element was shown by isolating plasmid-cured isogenic strains that had lost the plasmid with the IS*Aba825*-$bla_{OXA-58-like}$ genes but during that same process also lost the chromosomal IS*Aba825* element present upstream of the bla_{OXA-65} gene. A 16-fold decrease in minimum inhibitory concentration of imipenem and an eight-fold decrease in the minimum inhibitory concentration of meropenem were seen in the isogenic strains that lost the plasmid. The study presents the first report of IS*Aba825* simultaneously governing the bla_{OXA-65} gene and the $bla_{OXA-58-like}$ gene expression and also highlights the importance of this element in carbapenem-sensitive isogenic strains, which were once carbapenem resistant.

Keywords: *Acinetobacter baumannii*, β-lactamases, carbapenems, gene environment, insertion sequences
Original Submission: 22 February 2012; **Revised Submission:** 21 June 2012; **Accepted:** 24 June 2012
Editor: R. Cantón

Clin Microbiol Infect

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Introduction

Acinetobacter baumannii is an important opportunistic pathogen responsible for a variety of nosocomial infections [1]. Multidrug-resistant *Acinetobacter baumannii* causes serious infections associated with high mortality rates, including septicaemia, pneumonia and urinary tract infections, especially in intensive-care units because of the rapid evolution of this multi-drug-resistant pathogen when challenged by antibiotic therapy [2,3]. The emerging resistance to carbapenems among nosocomial strains represents a major concern worldwide [4,5].

The major mechanisms that play a significant role in carbapenem resistance in *A. baumannii* are the expression of OXA-type carbapenemases [1,3,4,6]. Other mechanisms, such as alterations in outer membrane permeability, can also contribute to carbapenem resistance in this organism [7–9]. Additionally, the disruption of the outer membrane channel CarO as a result of the natural insertional inactivation of its coding gene by insertion sequence (IS) *Aba825* or IS*Aba125* has been correlated with reduced susceptibility to carbapenems [8].

IS*Aba825*, a member of the IS*982* family (http://www-is.biotoul.fr), is composed of an 876-bp open reading frame and codes a DDE-type transposase bounded by a 17-bp inverted repeat [8]. It has been shown that IS*Aba825* generated a 7-bp duplication (ATCGTTA) at the insertion site within *carO* [8]. It is well known that insertion sequences can cause mutations and genome rearrangements to enhance the spread of resistance genes and virulence determinants within the pathogenic species [10–13]. A recent report by Ravasi *et al.* [14] has shown the association and the tight regulation of IS*Aba825* with the plasmid-borne bla_{OXA-58} gene. In this work, we evaluated the impact of IS*Aba825* in modulating

A. baumannii genome plasticity and carbapenem resistance and report for the first time IS*Aba825* modulating the bla_{OXA-51} gene expression simultaneously with the increased expression of the bla_{OXA-58} gene.

Materials and Methods

Four non-repetitive and distinct clinical strains, isolated in 2004 in the USA, were used in this study. They were confirmed as *A. baumannii* by PCR and sequencing of the *rpoB* gene using the primers Ac696F and Ac1093R described previously by La Scola *et al.* [15].

Minimum inhibitory concentration determination

The MICs of imipenem and meropenem were determined by the agar double-dilution method and the results were interpreted according to the British Society for Antimicrobial Chemotherapy guidelines [16]. The reference strains used for MIC testing were *Escherichia coli* NCTC 10418, *Pseudomonas aeruginosa* ATCC 10662 and *Staphylococcus aureus* NCTC 6571.

Screening of antimicrobial resistance determinants

The isolates were tested for the OXA group of enzymes by multiplex PCR as described by Higgins *et al.* [17]. Multiplex PCR for the detection of metallo-β-lactamases such as IMP, VIM, GIM, SIM and SPM was performed using the primers described previously by Ellington *et al.* [18].

The primers FxOxaF and FxOxaR, described earlier by Lopes *et al.* [19], were used for the detection of the presence of an insertion element upstream of the $bla_{OXA-51-like}$ gene in all four isolates, and the primers 51F and IntaceR described earlier [19] were used to map the OXA-51-like gene sequence with the screening for insertion sequence if any, present downstream from the OXA-51-like gene.

The primers preOXA-58prom^{+} and preOXA-58B, described previously [6] were used to check for any variations within the $bla_{OXA-58-like}$ gene by sequencing. The primer SM2 described previously by Poirel and Nordmann [20] and the walk-58-R primer described by Lopes *et al.* [19] were used to detect any insertion sequence upstream of the OXA-58-like gene.

Pulsed field gel electrophoresis

*Apa*I macrorestriction was performed on all four *Acinetobacter baumannii* strains according to the procedure described by Seifert *et al.* [21]. Cluster analysis was performed using the unweighted pair group method with mathematical averaging (UPGMA), and DNA relatedness was calculated by using the band-based Dice coefficient with a tolerance setting of 1.5% band tolerance and 1.5% optimization setting for the whole profile.

Gel analysis was performed using the BIONUMERICS v 2.5 software (Applied Maths, Sint-Martins-Latem, Belgium). A value of 85% was chosen as the threshold for the establishment of clonal relatedness of the strains.

Multilocus sequence typing

Multilocus sequence typing was performed according to the scheme proposed by Bartual *et al.* [22].

S1 nuclease digestion and plasmid determination

S1 nuclease digestion was performed for the pulsed field gel electrophoresis plugs according to the manufacturer's instructions (Promega, Southampton, UK). A total of 10 units per plug were used for digestion with incubation at 37°C/45 min. The plasmid bands were excised from the gel and purified using the Qiagen gel extraction kit. Chromosomal DNA contamination was checked using the 16S-rRNA primers described earlier by Lin *et al.* [23].

Plasmid curing

Plasmid curing was performed using an elevated temperature of incubation for which the strains were sub-cultured for 15 days at 46°C [24]. Plasmid curing was also performed using SDS: 200 μL of overnight culture was inoculated in 4.8 mL nutrient broth containing 200 μL of 10% SDS and incubated at 46°C/48 h [24]. Finally, SDS was coupled with elevated temperature of incubation to check for any loss of plasmid [24]. The strains (200 μL of overnight culture) were incubated in 4.8 mL nutrient broth with 200 μL 10% SDS at 46°C/48 h.

Conjugation

Transconjugation assays of *A. baumannii* strains with the $bla_{OXA-58-like}$ gene were performed with *E. coli* J62.2 and *A. baumannii* 19606 as described previously [25].

Analysis of gene expression

The gene expression was studied for all the isolates according to the method described previously by Lopes *et al.* [26]. The internal bla_{OXA-65} gene primers 65A (5′-CTCGTGC TTCGACCGAGTAT-3′) and 65B (5′-GCTGAACAACC CATCCAGTT-3′) were used for the bla_{OXA-65} gene expression and OXA-58-like F and OXA58-like R primers as described previously by Higgins *et al.* [17] were used for the bla_{OXA-58} gene expression. The PCR products were quantified using the BIO-RAD QUANTITY ONE Software 4.6.1 (Bio-Rad, Hercules, CA) (Table 1). Normalization of DNA was

TABLE 1. Activities of antibiotics and the levels of gene expression in clinical isolates of *A. baumannii*

	Minimum inhibitory concentration (mg/L)		Quantification of gene expression (arbitrary units)					
Strain*	Imipenem	Meropenem	Expression of the 16S rRNA gene		Expression of the $bla_{OXA-51-like}$ gene		Expression of the bla_{OXA-58} gene	
Ab202	16	16	16S r-RNA	249	OXA-65	154	OXA-58	194
Ab203	16	16	16S r-RNA	250	OXA-65	167	OXA-58	189
Ab204	16	16	16S r-RNA	255	OXA-65	165	OXA-58	187
Ab205	16	16	16S r-RNA	251	OXA-65	165	OXA-58	189
Ab202s	1	2	16S r-RNA	240	OXA-65	74	OXA-58	–
Ab203s	1	2	16S r-RNA	245	OXA-65	72.1	OXA-58	–
Ab204s	1	2	16S r-RNA	255	OXA-65	67.5	OXA-58	–
Ab205s	1	2	16S r-RNA	255	OXA-65	69	OXA-58	–
Ab3	0.25	0.5	16S r-RNA	245	OXA-95	51.1	OXA-58	71.8

–, denotes the absence of the bla_{OXA-58} gene.
*The strains 202, 203, 204 and 205 are cured strains from their respective parents.

performed with the 16S-rRNA primers described earlier by Lin *et al.* [23] with the appropriate amounts of serially diluted RNA used for cDNA synthesis.

Results

All the strains were resistant to imipenem and meropenem (Table 1). *Apa*I macrorestriction analysis of the four isolates exhibited genetic similarities of 99.99% by the unweighted pair group method with arithmetic average method (data not shown). All the isolates were found to have the sequence type ST114 (1, 15, 8, 10, 28, 57, 32) (http://pubmlst.org/abaumannii/, last accessed 15 June 2011). Genes encoding known carbapenemases were investigated as described previously and the genes $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ were detected in all the four strains [17]. Genes encoding OXA-40-like and OXA-23-like carbapenemases were not detected. Nor were metallo-β-lactamases such as IMP, VIM, GIM, SIM and SPM detected [18] in any of the isolates.

The primers FxOxaF and FxOxaR detected the presence of an insertion element upstream of the $bla_{OXA-51-like}$ gene in all four isolates (Fig. 1). The primers 51F and IntaceR were used for the amplification of the $bla_{OXA-51-like}$ gene and the intergenic region present downstream from the $bla_{OXA-51-like}$ gene and the phosphinothricin acetyltransferase (*GNAT*) gene (Fig. 1). The sequencing results showed the presence of IS*Aba825* upstream of the bla_{OXA-65} gene in all four isolates. A putative promoter was found with −35 (TTGTCA) and −10 (TATGAA) located 17 bp apart from each other (BPROM, Softberry, Inc., Mount Kisco, NY) located 97 and 74 bp upstream of the bla_{OXA-65} gene. A target site duplication of 7 bp, AAGTCTT was seen upstream and downstream of the IS*Aba825* sequence. No insertion was seen downstream of the bla_{OXA-65} gene making the IS*Aba825* a defunct transposon. The IS*Aba825* sequence was oriented in 5′–3′ direction against the bla_{OXA-65} gene in all four isolates.

The expression of the bla_{OXA-65} gene for all isolates was studied as described earlier by Lopes *et al.* [26] The internal bla_{OXA-65} gene primers 65A and 65B showed a considerable over-expression of the bla_{OXA-65} gene expression (Table 1).

Since the multiplex PCR detected the $bla_{OXA-58-like}$ gene, we further examined the isolates for any variations within the gene and insertion sequences involved in governing the expression of the $bla_{OXA-58-like}$ gene. The primers preOXA-58prom^{+} and preOXA-58B, described previously [6], were used to check for any variations within the $bla_{OXA-58-like}$ gene. The primers amplified a 934-bp fragment, which on sequencing confirmed that the bla_{OXA-58} gene had no nucleotide substitutions.

The SM2 primer and the walk-58-R primer amplified a fragment of IS*Aba825* oriented in 3′–5′ direction in all four parent strains. The putative promoter −35 (TTGAGA) present 148 bp upstream of the bla_{OXA-58} gene and −10 (TTTATA)

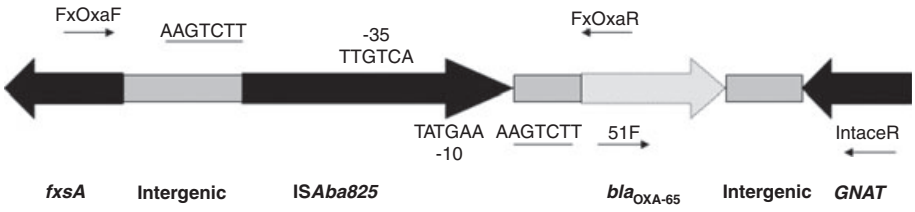

FIG. 1. Genetic arrangement of IS*Aba825* governing the chromosomal bla_{OXA-65} gene. The arrows represent the primers used for detection and sequencing of genes. Figure is not to scale.

present 127 bp upstream of the bla_{OXA-58} gene were identified 15 bp apart from each other. The bla_{OXA-58} gene was highly over-expressed (Table 1). The comparison was made with the bla_{OXA-58} gene of *A. baumannii* ATCC 17978/pWHP3 (Ab3) [14].

S1 nuclease digestion showed a plasmid of approximately 40 kb that harboured the bla_{OXA-58} gene in all the strains. The loss of plasmid was not observed when the conditions of elevated temperature or SDS were used individually—only when the two conditions were coupled together as described in the Materials and Methods. The loss of plasmid was observed in all the isogenic strains harbouring the IS*Aba825* element upstream of the bla_{OXA-58} gene, which was lost by the plasmid curing method as described in the Materials and Methods section. During the same process of curing, the IS*Aba825* that was present upstream of the bla_{OXA-65} gene was also lost, making the isolates carbapenem sensitive (Table 1). A 16-fold decrease in MIC of imipenem and an eight-fold decrease in MIC of meropenem were observed for all the sensitive isogenic strains. The isolates did retain the basal level of the bla_{OXA-65} gene expression (Table 1).

Transconjugation assays using *E. coli* J62.2 or *A. baumannii* 19606 could not transfer the 40-kb plasmid, which had the bla_{OXA-58} gene with the upstream IS*Aba825* element.

Discussion

Insertion sequence elements are capable of independent transposition within a genome and have evolved with mechanisms of regulation of genes in host cells [19]. Transposition of an IS element can either lead to the disruption of a gene causing insertional inactivation of a gene [19] or provide a signal for the activation, leading to the over-expression of the adjacent gene by providing suitable strong promoter sequences when present upstream of the gene [20]. The IS activation or inactivation depends on complexes formed by repressor–inhibitory mechanisms under antibiotic stress conditions and has an important role in regulation of genes [19]. The insertion of IS elements upstream of a β-lactamase gene provides the bacterium with the advantage of transiently increasing the levels of gene expression to combat antibiotic stress [19]. Transposition of the IS is therefore one of the most important driving forces that enhances the variability leading to a better evolutionary capacity and adaptability of their hosts [27]. This is demonstrated in our study by the acquisition of the plasmid-borne bla_{OXA-58} gene with IS*Aba825* and the loss of the same gene, leading to a sensitive phenotype in all the isogenic strains.

The curing event promoted the loss of the bla_{OXA-58} gene and its upstream IS*Aba825* promoter. However, the same event also promoted the loss of the IS*Aba825* element upstream of the chromosomally encoded bla_{OXA-65} gene, so making the strains carbapenem sensitive. These results suggest that control of the IS elements is well coordinated by the activators and repressors because the loss of the IS element occurred at the same step as the loss of the plasmid [19]. The transposition activity of an insertion element is usually present at low levels because the change caused by the increase in activities can have a detrimental effect on the host cell through various modifications in the genome [27]. The transposase promoters are usually located in the terminal inverted repeats, and are generally autoregulated; this would enable the bacterium to resist antibiotic stress without compromising its fitness cost [28].

The temperature sensitivities of transposition in *E. coli* have been identified as the properties of the transposases, which are inherently present within the genome and which contribute towards genomic variability under critical growth conditions [29]. It has been reported earlier that an increase in the transposition activities of the seven IS elements belonging to four different IS families of *Burkholderia multivorans* ATCC 17616 suggested that there may be some common host factors that affect the transposition frequency at high temperatures and so lead to genome rearrangement [27]. Ohtsubo *et al.* [27] also suggested that there may be other factors that could be responsible for changes in the genome and could either promote or inhibit the effective working of transposases. The observed characteristics for the carbapenem-resistant and carbapenem-sensitive strains indicate that the IS element examined here (IS*Aba825*) plays an important role in cells exposed to high temperatures, so generating genetic diversity in a population of *A. baumannii*. High temperatures may be important for *A. baumannii* because it is a frequent pathogen in the Arabian Peninsula and appears to be present in the hospital environment there, where temperatures frequently reach 46°C. Expression studies also show the importance of IS*Aba825* in conferring carbapenem resistance by over-expression of the $bla_{OXA-58-like}$ and bla_{OXA-65} genes and it can be concluded that IS*Aba825* is also crucial for the adaptation and evolution of carbapenem-resistant *A. baumannii* besides the well-known IS*Aba1* [30]. Our results concur with those of Ravasi *et al.* [14] and additionally we indicate that IS*Aba825* also governs the $bla_{OXA-51-like}$ gene expression and this is an essential mechanism of carbapenem resistance in *A. baumannii*. Regardless of whether the β-lactamase gene was on a plasmid, it is important to note that IS*Aba825*, present upstream of the bla_{OXA-65} gene, can easily provide a stable mechanism

of resistance if the strain is under antibiotic pressure. When the strain is subjected to unfavourable conditions in the absence of any antibiotic stress, such as elevated temperature or other chemicals like SDS, the loss of plasmid or the elimination of chromosomal IS*Aba825* present upstream of the bla_{OXA-65} gene favours survival as the bacterium no long needs to express the resistance genes. This presumably reduces the energy cost on the cell. These results also highlight that gene duplication being crucial in multi-resistant strains of *A. baumannii* does involve recombination events involving insertion elements that play an important role in the adaptation of the bacterium. This adaptation of the bacterium to survive under unfavourable conditions of high antibiotic pressure or other unsuitable physiological conditions clearly demonstrates the adaptation and the resilient nature of *A. baumannii*. The insertion element IS*Aba825* belongs to the IS*982* family, once thought to be restricted to Gram-positive bacteria [28], this denotes the interspecific spread of insertion elements between different bacterial communities. As IS*Aba825* was previously reported to be plasmid-borne in *A. baumannii* from Argentina, we believe that the association and establishment of this element with the chromosomal β-lactamase ($bla_{OXA-51-like}$) of *A. baumannii* should be of great concern [14]. It is worth noting that the sequence type ST114 (1, 15, 8, 10, 28, 57, 32) found in our strains has been previously described in other isolates from Argentina (http://pubmlst.org/abaumannii/, last accessed 15 June 2011).

Nucleotide sequence accession number

The IS*Aba825*-bla_{OXA-58} sequence of strain 202 has been deposited under the accession number JQ412186 and the IS*Aba825*-$bla_{OXA-51-like}$ sequence of the same strain has been deposited under the accession number JQ412185.

Acknowledgements

We would like to thank MRC for grant number RA0119, which funded part of this work. We are also grateful to the University of Edinburgh for the overseas research scholarship to BSL. We are grateful to Dr Mussi for providing us with Ab 17978/pWHP3 isolate. A part of this work was presented at the 51 Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 2011.

Transparency Declaration

None to declare.

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ORIGINAL ARTICLE 10.1111/1469-0691.12143

Diversity in *Acinetobacter baumannii* isolates from paediatric cancer patients in Egypt

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Abstract

Acinetobacter baumannii is an important nosocomial pathogen, commonly causing infections in immunocompromised patients. It is increasingly reported as a multidrug-resistant organism, which is alarming because of its capability to resist all available classes of antibiotics including carbapenems. The aim of this study was to examine the genetic and epidemiological diversity of *A. baumannii* isolates from paediatric cancer patients in Egypt, by sequencing the intrinsic $bla_{OXA-51-like}$ gene, genotyping by pulsed-field gel electrophoresis and multilocus sequence typing in addition to identifying the carbapenem-resistance mechanism. Results showed a large diversity within the isolates, with eight different $bla_{OXA-51-like}$ genes, seven novel sequence types and only 28% similarity by pulsed-field gel electrophoresis. All three acquired class-D carbapenemases (OXA-23, OXA-40 and OXA-58) were also identified among these strains correlating with resistance to carbapenems. In addition, we report the first identification of IS*Aba2* upstream of $bla_{OXA-51-like}$ contributing to high-level carbapenem resistance. This indicates the presence of several clones of *A. baumannii* in the hospitals and illustrates the large genetic and epidemiological diversity found in Egyptian strains.

Keywords: *Acinetobacter baumannii*, $bla_{OXA-51-like}$, carbapenem-hydrolysing class D β-lactamase, diversity, insertion sequences, IS*Aba2*., resistance

Original Submission: 2 October 2012; **Revised Submission:** 28 November 2012; **Accepted:** 28 December 2012

Editor: R. Cantón

Clin Microbiol Infect

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Introduction

Acinetobacter baumannii has emerged as an important nosocomial pathogen in the past decade, which in recent years has developed into a multidrug-resistant problematic pathogen [1]. *Acinetobacter baumannii* is an opportunistic pathogen, frequently isolated from immunocompromised patients with prolonged hospitalization [2]. As a consequence of immunoablative treatment, patients with cancer are at risk of developing *A. baumannii* infections, including sepsis, respiratory, wound and tissue infections, in addition to urinary tract infections [2,3].

A major concern in *A. baumannii* is its worldwide clonal expansion and its ability to survive and disseminate in hospitals, with numerous outbreaks reported from different regions of the world [4]. *Acinetobacter baumannii* is notably resistant to extreme environmental conditions, such as dryness, and can survive on surfaces for a long time, hence facilitating its spread [1,4].

Resistance to carbapenems, the β-lactam drugs of last resort in treating *A baumannii* infections, has been attributed to the expression of carbapenem-hydrolysing oxacillinase genes, bla_{OXA23}, bla_{OXA-40} and bla_{OXA58}, which are usually plasmid encoded [5,6]. The ubiquitous, chromosomally encoded $bla_{OXA-51-like}$ gene only confers resistance when an Insertion Sequence (IS) is present upstream of the gene [7].

Due to the prevalence of *A. baumannii* across the world, suitable typing methods to investigate the epidemiological distribution of the organism have been developed such as ribotyping, amplified fragment length polymorphisms, pulsed-field gel electrophoresis (PFGE) and, more recently,

Multi-Locus Sequence Typing (MLST) [8]. Additionally, amplification and sequencing of the ubiquitous $bla_{\text{OXA-51-like}}$ gene has also been used to determine clonal groups from diverse worldwide sources [7,8].

Limited data were available concerning the epidemiological distribution of *A. baumannii* in the Middle East but, in the past few years, reports of strains in the United Arab Emirates, Iraq, Kuwait and Egypt harbouring diverse resistance mechanisms have emerged [9–12]. The aim of this study was to investigate the epidemiological and molecular diversity of *A. baumannii* strains isolated from two cancer centres in Cairo, Egypt.

Materials and Methods

Isolate identification

Thirty-four non-duplicate *A. baumannii* were obtained from two centres; The Children's Cancer Hospital (CCH) and The National Cancer Institute (NCI), both located in Cairo, Egypt, from 2010 to 2011. Initial identification and susceptibility testing was done using VITEK and Phoenix automated machines. Genotypic identification was carried out by restriction analysis of 16s-23s rRNA spacer sequences using *Alu*I and *Nde*II [13].

Detection of $bla_{\text{OXA-51-like}}$ genes

The intrinsic $bla_{\text{OXA-51-like}}$ genes were amplified for *A. baumannii* isolates using primers: OXA69A and B [7]. Products were purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and sequenced in both directions on a 3730 DNA Analyzer (Applied Biosystems, Warrington, UK). For isolates yielding a larger product size, a PCR was performed to screen for the associated upstream environment using primers FxOxa-F and FxOxa-R [14].

Detection of class D oxacillinases and genetic environment

Isolates were screened for the presence of acquired OXA carbapenemases by Multiplex PCR, as previously described [15]. Isolates positive for the individual OXA groups were subsequently amplified and sequenced using primers for the full sequence of the genes. Associated genetic environment was also amplified and sequenced. Primers used are listed in Table 1.

Minimum inhibitory concentrations

The MIC of imipenem and meropenem were determined using an agar double dilution technique according to the British Society of Antimicrobial Chemotherapy (BSAC) guidelines [16]. *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 were used as control strains.

Pulsed-field gel electrophoresis

All isolates were typed by PFGE according to the procedure previously described by Seifert *et al.* [17]. Briefly, plugs were incubated in 30 U *Apa*I at 37° overnight, and subsequently run on 1% pulsed-field-certified agarose gel (Bio-Rad, Hertfordshire, UK) in 0.5 × TBE buffer with an initial pulse of 5 s and a final pulse of 20 s for 20 h. The gels were stained with Gel-Red solution and visualized using the DIVERSITY DATABASE (Bio-Rad) software image-capturing system.

Multi-locus sequence typing

The PCR for the seven housekeeping genes: *gltA*, *gyrB*, *gdhB*, *rpoD*, *recA*, *gpi* and *cpn60* was performed according to the scheme developed by Bartual *et al.* [18]. Products were purified and sequenced as described above. MLST was performed for ten isolates, representatives of the $bla_{\text{OXA-51-like}}$ gene variants identified. If isolates from different hospitals harboured similar $bla_{\text{OXA-51-like}}$ genes, an isolate from each hospital was selected randomly for comparison. Isolates chosen for MLST were: 8357, 9925-SAM, 1780, 634, 21174, 22055, 161, P38-YSF, P67-AZ and 14611.

TABLE 1. List of primers used in this study

Primer name	Sequence 5′–3′	Use	Reference
16s-23s rRNA F	TTGTACACACCGCCCGTCA	Identification	[13]
16s-23s rRNA R	GGTACTTAGATGTTTCAGTTC		
Oxa69-A	CTAATAATTGATCTACTCAAG	$bla_{\text{OXA-51-like}}$ amplification and sequencing	[7]
Oxa69-B	CCAGTGGATGGATGGATAGATTATC		
FxOxaF	GATACCAGACCTGGCAACAT	Upstream environment of $bla_{\text{OXA-51-like}}$ gene	[14]
FxOxaR	GCACGAGCAAGATCATTACC		
$bla_{\text{OXA-23}}$ F	GATGTGTCATAGTATTCGTCG	Whole gene-sequence of bla_{OXA23}	[25]
$bla_{\text{OXA-23}}$ R	TCACAACAACTAAAAGCACTG		
ISAba1A	GTGCTTTGCGCTCATCATGC	Upstream environment of bla_{OXA23}	[26]
SM2	AAGTGTCTATATTCTCACC	Upstream environment of bla_{OXA58}	
ISAba3-F	CAATCAAATGTCCAACCTGC	Upstream environment of bla_{OXA58}	
OXA-58A	CGATCAGAATGTTCAAGCGC	Whole gene sequence of bla_{OXA58}	[22]
OXA-58B	ACGATTCTCCCCTCTGCGC		
OXA-24FF	ATGAAAAAATTTATACTTCCTA TATTCAGC	Whole gene sequence of bla_{OXA24}	[27]
OXA-24RR	TTAAATGATTCCAAGATTTTCTAGC		

Results

Diversity of *bla*$_{OXA-51-like}$ genes

All isolates were confirmed as *A. baumannii*, and sequencing of the intrinsic *bla*$_{OXA-51-like}$ revealed the presence of eight different genes: *bla*$_{OXA-64}$, *bla*$_{OXA-65}$, *bla*$_{OXA-66}$, *bla*$_{OXA-69}$, *bla*$_{OXA-71}$, *bla*$_{OXA-78}$, *bla*$_{OXA-94}$ and *bla*$_{OXA-89}$ (Table 2). *bla*$_{OXA-65}$ was the most prevalent, found in 14 isolates, obtained from both hospitals. *bla*$_{OXA-64}$ is now commonly found in the Middle East (A. Al Hasan, and S.G.B. Amyes, unpublished results; [9]), it was found in seven isolates obtained from both hospitals. There were representatives from the three worldwide clones (formally known as the European clones). *bla*$_{OXA-66}$ was found in four isolates, three of which were from CCH. *bla*$_{OXA-69}$ was identified in two isolates at the intensive care unit (ICU) of CCH and were part of an *A. baumanniii* outbreak in early 2011. *bla*$_{OXA-71}$ was found in two isolates from different hospitals. *bla*$_{OXA-78}$ and *bla*$_{OXA-89}$ were both found in strains from CCH, whereas *bla*$_{OXA-94}$ was from two isolates from NCI, recovered from the same floor, 1 day apart.

Insertion sequences associated with *bla*$_{OXA-51-like}$

Sequencing upstream of the *bla*$_{OXA-51-like}$ gene, *bla*$_{OXA-89}$ in isolate 22055 revealed the presence of IS*Aba2*, with the -35 (ttatat) and -10 (ttgtaggat) promoters 29 bp apart, and located 102 bp and 82 bp upstream of *bla*$_{OXA-89}$, respectively. No other insertion sequences were identified upstream of the *bla*$_{OXA-51-like}$ genes.

PFGE

The PFGE analysis revealed a large diversity within the strains. Some isolates with similar *bla*$_{OXA-51-like}$ genes had very distinct PFGE patterns, suggesting no epidemiological similarity between the strains. As seen in Figure 1, only six isolates harbouring *bla*$_{OXA-65}$ show > 80% similarity in their PFGE pattern. Additionally, *bla*$_{OXA-64}$ isolates all shared less than 80% similarity. Even isolates with *bla*$_{OXA-94}$, which were collected from patients on the same floor of the same hospital 1 day apart, had distinct PFGE patterns. On the other hand, the *bla*$_{OXA-71}$ containing isolates, although from different hospitals, had similar PFGE patterns. The similarity for all the isolates was calculated by Dice coefficient to be 28.7%.

TABLE 2. Isolates harbouring *bla*$_{OXA-51-like}$ genes, with isolation details. carbapenem-hydrolysing class D β-lactamase (CHDL) genes, minimum inhibitory concentration (MIC) and sequence type. Isolates in bold were in the *A. baumannii* outbreak in early 2011

Isolation details					CHDL β-lactamase gene				MIC (mg/L)		
Isolate no.	Date of sample	Hospital	Location	Site of isolate	*bla*$_{OXA-51-like}$	*bla*$_{OXA-23}$	*bla*$_{OXA-58}$	*bla*$_{OXA-40}$	IMI	MER	Sequence type
7947	17/05/2010	CCH	ICU	Wound	64				0.25	0.06	
12435	23/07/2010	CCH	ICU	Blood	64	+			16	32	
14298	22/08/2010	CCH	ICU	Catheter tip	64		+		8	2	
8357	5/29/2011	CCH	DSCH	CVP Blood	64				8	16	ST408
4248	15/03/2010	CCH	IP-5C	CVP tip	65	+			8	4	
4842	23/03/2010	CCH	ICU	CVP tip	65	+			64	32	
9930	15/06/2010	CCH	ICU	Blood and CVP	65	+			32	16	
10262	19/06/2010	CCH	ICU	CVP	65	+			64	128	
15094	05/09/2010	CCH	IP-4B	Urine	65				0.25	0.12	
15324	09/09/2010	CCH	IP-3A	Catheter tip	65				0.06	0.06	
1780	31/01/2011	CCH	ICU	Stool	65	+			8	16	ST410
1750	31/01/2011	CCH	ICU	BAL	65	+			32	8	
2106	08/02/2011	CCH	ICU	Blood	65	+			8	8	
2632	20/02/2011	CCH	IP-5A	Stool	65	+			8	8	
2625	20/02/2011	CCH	IP-4C	Urine	65	+	+		16	16	
8768	6/4/2011	CCH	IP-4B	Blood	65				0.008	0.03	
4343	17/03/2010	CCH	IP-3B	CVP-Blood	66				8	16	
14611	27/08/2010	CCH	ICU	CVP Blood	66			+	64	32	ST208
21382	13/12/2010	CCH	ICU	CVP Culture	66	+			16	4	
7052	5/7/2011	CCH	ICU	BAL	66	+			8	16	
634	11/01/2011	CCH	ICU	Catheter tip	69	+			8	4	ST108
1447	24/01/2011	CCH	ICU	CVP Culture	69	+			0.5	0.06	
161	03/01/2011	CCH	PULM	Sputum	71				0.06	0.06	ST414
21174	09/12/2010	CCH	IP-3A	Blood	78				0.03	0.25	ST412
22055	25/12/2010	CCH	IP-3C	CVP Blood	89				128	128	ST413
679-BAS	04/09/2010	NCI	5th floor	Ear swab	64		+		8	8	
P67-AZ	09/01/2011	NCI	OP	Blood	64	+	+		64	16	ST411
9925-SAM	15/12/2010	NCI	7th floor	Blood	64	+			16	8	ST409
P391-AH	14/09/2010	NCI	5th floor	Blood	65				8	16	
461-SF	15/12/2010	NCI	7th floor	Blood	65	+			16	32	
6332-ABD	02/09/2010	NCI	5th floor	Ear swab	69	+			16	8	
5687-SHAY	11/10/2010	NCI	5th floor	Blood	71				0.25	0.25	
P38-YSF	04/01/2011	NCI	5th floor	Blood	94	+			64	64	ST331
P49-HAM	05/01/2011	NCI	5th floor	Blood	94		+		8	8	

BAL, bronchoalveolar lavage; CVP, central venous port; IMI, imipenem; MER, meropenem.

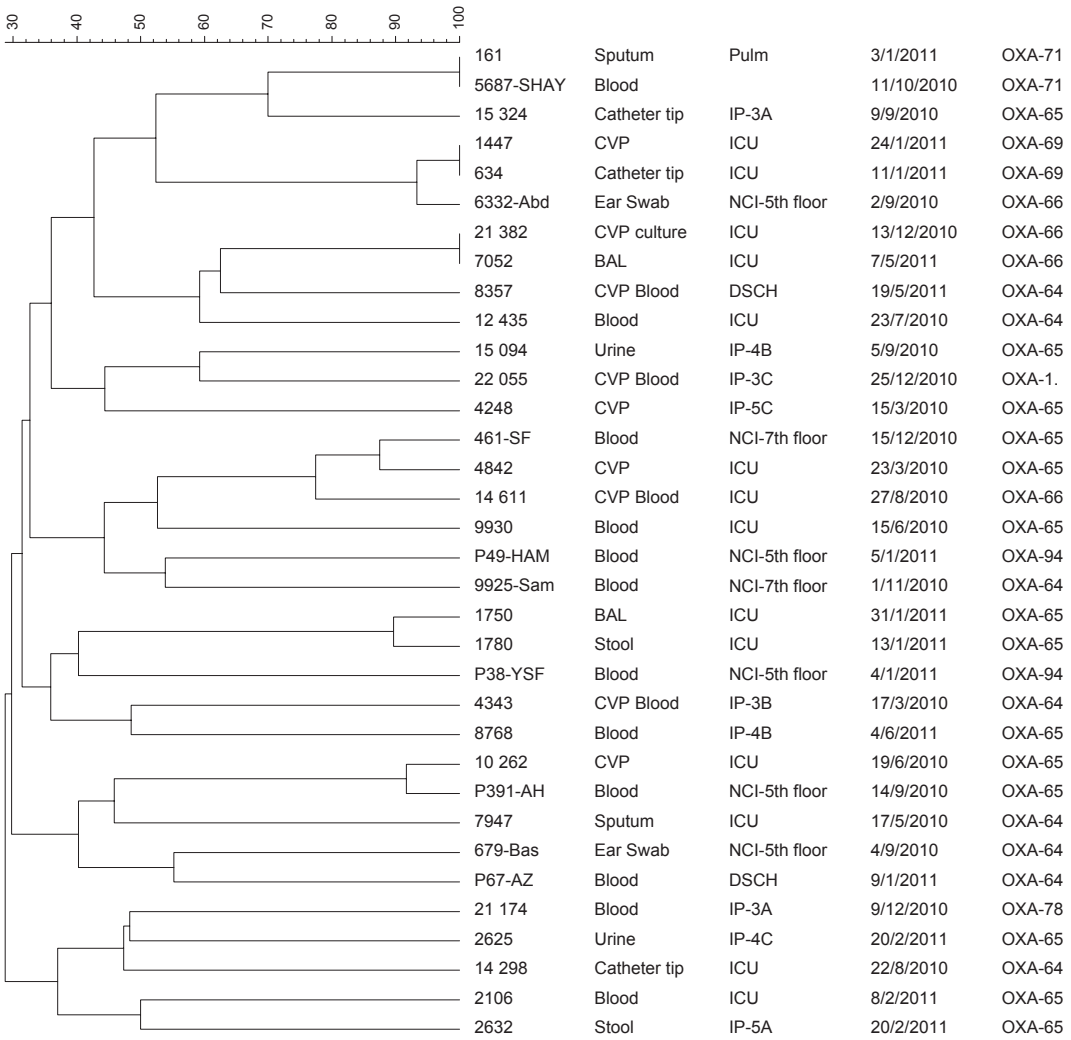

FIG. 1. Pulsed field gel electrophoresis profile for *Acinetobacter baumannii* strains in this study, showing the associated isolation site, location, date, and $bla_{OXA51\text{-like}}$ genes.

MLST

Seven housekeeping genes were amplified and sequenced as described above for ten isolates. Ten distinct sequence types (STs) were identified, seven of which are novel and assigned ST408–ST414. The remaining three STs were identified as ST331, ST108 and ST208. Typing by MLST further illustrated the large diversity found within the strains, as isolates with similar $bla_{OXA\text{-}51\text{-like}}$ genes had different STs. This is clear for isolates 9925-SAM and NCI-P67, both were from the NCI and possessed $bla_{OXA\text{-}64}$, but they belonged to different STs: 409 and 411, respectively. When compared with another $bla_{OXA\text{-}64}$-positive isolate, 8357, which was from a patient at CCH, another ST was identified, ST408.

MIC and carbapenem-hydrolysing class D β-lactamase (CHDL) genes

The majority of isolates ($n = 25$), representing 73%, were resistant to imipenem and/or meropenem (MIC ≥ 8 mg/L). This resistance could be correlated with the presence of the acquired class-D oxacillinases: $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}58}$ and $bla_{OXA\text{-}40}$ (Table 2).

Genes encoding all three transferable OXA types associated with resistance were identified in these strains: $bla_{OXA\text{-}23}$ in 18 isolates, $bla_{OXA\text{-}58}$ in five isolates and $bla_{OXA\text{-}40}$ in one isolate. All isolates, except one, possessing $bla_{OXA\text{-}23}$ were resistant to imipenem and meropenem (MIC ≥ 8 mg/L). IS*Aba1* was detected upstream of $bla_{OXA\text{-}23}$ in the resistant isolates, hence providing a promoter for the expression of the gene (Figure 2). However, this IS element was not found upstream in the $bla_{OXA\text{-}23}$-containing isolate that was carbapenem sensitive. The analysis of the *A. baumannii* outbreak in the ICU at CCH in early 2011 revealed that although the strains harboured distinct $bla_{OXA\text{-}51\text{-like}}$ types and were epidemiologically different, they all possessed $bla_{OXA\text{-}23}$ as the resistance mechanism.

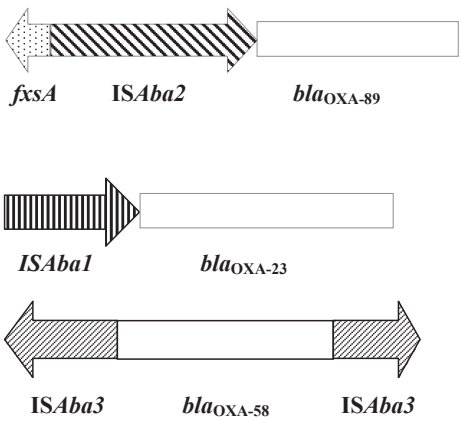

FIG. 2. Schematic representation showing examples of the genetic environments of bla_{OXA-89}, bla_{OXA-23} and bla_{OXA-58}.

bla_{OXA-58}-positive isolates were also found in both hospitals and all were resistant to meropenem and imipenem, with the exception of isolate 14298, which was intermediate to meropenem (MIC 4 mg/L). The genetic environment of the bla_{OXA-58} showed that the gene was flanked by two copies of IS*Aba3* (Figure 2). Two isolates harboured an interrupted sequence of IS*Aba3* upstream of the bla_{OXA-58} gene (L. Al-Hassan, H. El Mehallawy and S. G. B. Amyes, unpublished results).

A single isolate, 14611 from CCH, was positive for bla_{OXA-40} and it was also resistant to carbapenems. No insertion element was detected upstream of the bla_{OXA-40} gene.

Eight of the 11 isolates that did not harbour acquired carbapenemase genes were sensitive to carbapenems (MIC <8 mg/L). One isolate, 22055, lacking these genes was resistant to carbapenems and harboured the chromosomal OXA-89 β-lactamase. IS*Aba2* was found upstream of the bla_{OXA-89} gene (Figure 2).

Discussion

Acinetobacter baumannii is a problematic, multidrug-resistant pathogen identified in healthcare environments worldwide [1]. The remarkable ability of *A. baumannii* to capture and express resistance genes has allowed it to become one of the major threats in hospitals, as it becomes resistant to all available antibiotics, including carbapenems [4]. Resistance mechanisms such as modification of target site, efflux pumps and enzymatic inactivation have all been reported in *A. baumannii* [1]. Of major concern is the presence of several classes of β-lactamases within the *A. baumannii* genome. The localization of these resistance genes on plasmids facilitates their movement from one bacterium to another [5]. Class D oxacillinase genes: bla_{OXA-23}, bla_{OXA-40} and bla_{OXA-58} have been repeatedly reported in *A. baumannii* outbreaks from different parts of the world [1,19].

The construction of a linkage map based on the intrinsic OXA-51-like β-lactamases was reported by Evans *et al.* [7]. The sequence relationship was determined for 37 distinct members of the OXA-51-like β-lactamase family. This study identified three large groups around OXA-66, OXA-69 and OXA-98 in addition to other unrelated branched enzymes [7]. In the current study a large diversity was found in the sequences of $bla_{OXA-51-like}$ with eight different gene variants identified. This is particularly interesting given the short duration of isolate collection (1 year) as well as the isolates deriving from only two hospitals. In fact seven different $bla_{OXA-51-like}$ genes were identified in CCH alone. When looking at the distribution of $bla_{OXA-51-like}$ genes in the linkage map, it is clear that they have different origins as the genes identified are not clustered in closely related groups. Fourteen isolates, accounting for 41%, harboured bla_{OXA-65}, which according to the linkage map forms a 'central hub' from which all other groups radiate and is thought to be ancestral to all $bla_{OXA-51-like}$ genes [7]. This subsequently indicates the presence of the potential ancestral $bla_{OXA-51-like}$ gene in *A. baumannii* in Egypt, which is in the current collection of strains and is the major gene identified. Additionally, this may explain that the large diversity found is an outcome of the evolution of the ancestral bla_{OXA-65} gene in some cases, rather than the of 'foreign carriage' of clones into the country.

bla_{OXA-69}, bla_{OXA-66} and bla_{OXA-71} have been associated with Worldwide [European] Clones I, II and III, respectively, and all have been identified in the current study [6,7]. bla_{OXA-66} and bla_{OXA-71} genes were identified in both hospitals, which may indicate local distribution in Egyptian hospitals. bla_{OXA-69}, on the other hand, was found in two isolates in the ICU outbreak in early 2011 at CCH only. This illustrates the extent of spread of the major lineages of *A. baumannii*.

bla_{OXA-89} is a member of the bla_{OXA-98} cluster and contains the resultant protein showing three amino acid substitutions from OXA-98. In the current study, one isolate from CCH was found positive for bla_{OXA-89}, and harboured IS*Aba2* upstream. The presence of an insertion sequence upstream of other $bla_{OXA-51-like}$ genes has been reported to enhance the expression and cause resistance to carbapenems [20, 21]. IS*Aba2* has only been reported upstream of bla_{OXA58} [22]. With no other resistance mechanism identified, the presence of IS*Aba2* was responsible for high-level resistance to both imipenem and meropenem (MIC 128 mg/L and 256 mg/L, respectively). Furthermore, this shows the ability of IS to insert upstream of these genes and act as promoters.

bla_{OXA} genes that are not part of previously identified clusters have also been identified in the current study: bla_{OXA-94} in two

isolates from the NCI and bla_{OXA-64} in eight isolates from both hospitals. OXA-64 is closely related to OXA-71 and is now commonly found in the Middle East [7, 9] (A. Al-Hasan and S.G.B. Amyes, unpublished results). bla_{OXA-94}, on the other hand, forms a branch of bla_{OXA-65} cluster with three amino acid substitutions in the resultant protein.

As expected from this large diversity of isolates, there is considerable variation in their PFGE profiles. Notably, isolates harbouring similar $bla_{OXA-51-like}$ genes have different PFGE profiles and no epidemiological linkage can be inferred. This could be a result of the localization of the patients in different wards and at different times in the hospital. Even for isolates recovered from the ICU at different times, there seems to be significant variability in profiles suggesting the presence of different clones within the same hospital. Turton *et al.* found a correlation between PFGE and sequence typing, in contrast to Evans *et al.* who later noted major differences between PFGE typing and sequence typing in their study [7,23].

MLST further illustrated the diversity within the isolates as eight out of ten isolates typed were assigned to novel STs. Previous reports have shown that typing with $bla_{OXA-51-like}$ was more consistent with MLST than with PFGE [8]. In the current study, isolates 8357, P67-AZ and 9925-SAM had similar $bla_{OXA-51-like}$ genes but, when they were typed with MLST, they showed three different novel STs, 408, 409 and 411, respectively. The PFGE patterns were also different for these isolates. This could indicate the presence of three distinct clones in the two hospitals, especially that they were isolated in different months and in different wards. MLST, in this case, correlated with the epidemiological data of PFGE. Hamouda *et al.* [8] found MLST to be more accurate than PFGE when studying isolates on a global scale.

Seventy-three percent of the isolates were resistant to carbapenems, and this is associated with all three CHDL genes found in this study. Different genetic structures are associated with the upstream environment of bla_{OXA-58} and bla_{OXA-23} and they have been identified in different regions of the world [22,24]. In the current study, bla_{OXA-23} is associated with IS*Aba1* in the upstream environment and bla_{OXA-58} is flanked by IS*Aba3*. The effective mobilization of these genes by insertion sequences upstream together with the localization on plasmid largely contribute the spread of these resistance genes [4].

In conclusion, the data presented show the large diversity of *A. baumannii* isolated from two centres in Cairo, Egypt. The genetic plasticity of *A. baumannii* is represented by the presence of several insertion sequences upstream of the resistance genes, thereby facilitating the expression and causing resistance to carbapenems. Several clones seem to be present in Egyptian hospitals requiring increased awareness of the healthcare personnel and stricter infection control policies to prevent the dissemination of these isolates.

Nucleotide Sequence Accession Number

The IS*Aba2*-bla_{OXA-89} sequence of strain 22055 has been deposited under the accession number JX499236.

Acknowledgement

We are grateful to the hospital staff at The Children's Cancer Hospital, Egypt and The National Cancer Institute for providing us with the samples and allowing part of the work to be undertaken at their centres.

A part of this work was presented at the 22nd European Congress of Clinical Microbiology and Infectious Diseases, London, 2012.

Transparency Declaration

None to declare.

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APPENDIX B

Abstracts of conference posters and presentations

Identification of IS*1006* interrupting an IS*Aba3* upstream of bla_{OXA-58} in *Acinetobacter baumannii* from a cancer patient in Egypt.

L. Al-Hassan, H. El Mahallawy, B.S. Lopes, S.G.B. Amyes

Objectives:
A. baumannii is an important opportunistic infection that is commonly found in hospitals, particularly intensive care units. Class D carbapenamase bla_{OXA-58} is increasingly identified in *A. baumannii* as plasmid-mediated and is believed to confer carbapenem resistance when associated with insertion sequences (IS) upstream. We hereby report a carbapenem-sensitive *A. baumannii* strain isolated from a paediatric cancer patient in Egypt, harbouring bla_{OXA-58} with IS*1006* interrupting an IS*Aba3* upstream.
Materials and Methods:
The isolate was obtained from a catheter tip culture of a paediatric cancer patient in Cairo, identified by 16s-23s rRNA restriction analysis as well as by amplification and sequencing of the $bla_{OXA-51-like}$ gene. The presence of Class D Carbapenamases was performed by Multiplex PCR, and the upstream region of bla_{OXA-58} was amplified and sequenced accordingly. Minimum inhibitory concentrations (MICs) were determined according to BSAC guidelines
Results:
The isolate possessed the chromosomal bla_{OXA-64} gene, now commonly found in the Middle East. In addition, it was also positive for $bla_{OXA-58,}$. Sequencing directly upstream of bla_{OXA-58} revealed the presence of IS*Aba3*, which would normally provide a suitable promoter and be associated with carbapenem resistance. However, the MICs of Imipenem and Meropenem were 4 and 1 mg/L, respectively. Sequencing also revealed that the IS*Aba3* was interrupted by the incorporation of another insertion sequence, IS*1006*, 176 bp upstream of the start codon of $bla_{OXA-58,}$ which is interfering with the promoter of IS*Aba3*.
Conclusion:
IS*Aba3* has been previously reported upstream of bla_{OXA-58} conferring carbapenem resistance in *A. baumannii*. Our results suggest that the insertion of IS*1006* in the IS*Aba3* serves to switch off the resistance promoter, hereby rendering the isolate susceptible to carbapenems.

Diversity in *Acinetobacter baumannii* isolates from paediatric cancer patients in Egypt.

L. Al-Hassan, H. El Mehallawy, S.G.B. Amyes

Objectives:
Cancer patients are at a higher risk from serious infections because they are immuno-compromised and many causative organisms are multi-drug resistant. In this study we report the genetic and epidemiological diversity of *Acinetobacter baumannii* isolated from paediatric cancer patients in Egypt, an emerging problem in cancer centres.
Materials and Methods
Thirty-four *Acinetobacter baumannii* strains were collected from the Children's Cancer Hospital (CCH) 57357 and the National Cancer Institute (NCI) in Cairo from March 2010 - June 2011. They were initially identified phenotypically and then genotypically by PCR amplification and sequencing of $bla_{\text{OXA-51-like,}}$ and restriction analysis of 16s-23s rRNA spacer sequences using *AluI* and *NdeII*. Minimum inhibitory concentrations (MIC) of imipenem and meropenem was performed and interpreted according to BSAC guidelines. Isolates were also screened for the presence of class D carbapenamases: $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$ by PCR amplification and sequencing.
Results:
Sequencing of $bla_{\text{OXA-51-like}}$ gene revealed a large diversity among the strains with 8 different genes identified: $bla_{\text{OXA-64}}$, $bla_{\text{OXA -65}}$, $bla_{\text{OXA-66}}$, $bla_{\text{OXA-69}}$, $bla_{\text{OXA-71}}$, $bla_{\text{OXA-78}}$, $bla_{\text{OXA-94}}$ and $bla_{\text{OXA-100.}}$ This large diversity showed the presence of the 3 major sequence groups ($bla_{\text{OXA-66,}}$ $bla_{\text{OXA-69,}}$ and $bla_{\text{OXA-71}}$) in addition to other unrelated clones. Overall carbapenem resistance was 47% in all isolates (MIC ≥8mg/L), with resistance to meropenem being slightly higher than Imipenem in most strains. All three class D carbapenamases were detected in the isolates, with $bla_{\text{OXA-23}}$ being most common (18 isolates), whereas 7 isolates harboured the $bla_{\text{OXA-58}}$ and 2 isolates had $bla_{\text{OXA-40.}}$ Seven $bla_{\text{OXA-23}}$ isolates had IS*Aba1* inserted upstream and this correlated with higher resistance to carbapenems. Two isolates had the unusual combination of both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$.
Conclusion:
The two Egyptian hospitals are tertiary referral centres and these results show that the *A. baumannii* isolated from their patients had diverse origins although they included the 3 major European clones. The data strongly suggest that many of the strains have been brought to the hospital by the patients themselves rather than acquired by the more usual method of transmission through patient-to-patient cross infection. The results also show how these patients can serve as reservoirs for the survival and eventual dissemination of *A. baumannii*.